PROGRAM

1975 SAES COLLABORATORS WORKSHOP
Southern Regional Research Center
March 13-14, 1975

Dr. John W. Sites
Discussion Leader

THEME: "MYCOTOXINS AND FOOD SAFETY"

March 13, 1975 - 9:00 a.m.

Welcome and Presentation of comments by Harry W. Hays of the National Program Staff, ARS, USDA, Beltsville, Maryland	Mary E. Carter, Director, SRRC
Response	William P. Flatt, Director Agricultural Experiment Station University of Georgia Athens, Georgia

Session I

METHODOLOGY

Identification of Toxigenic Fungi	Urban L. Diener Professor of Plant Pathology Department of Botany and Microbiology Auburn University Auburn, Alabama
Comments on mycotoxin studies at LSU	Rosamond Lackett Killebrew Microbiologist LSU, Agricultural Experiment Station Baton Rouge, Louisiana
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Highly Sensitive Methods for Determining Aflatoxins	Walter A. Pons, Jr., Research Chemist Oilseed and Food Laboratory, SRRC
Aspergillus Toxins Other Than Aflatoxins	Richard J. Cole Research Microbiologist National Peanut Research Laboratory ARS, USDA Dawson, Georgia

Mycotoxin Research at VPI	David G. I. Kingston Department of Chemistry Virginia Polytechnic Institute and State University Blacksburg, Virginia

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March 14, 1975 - 9:00 a.m.

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RAW AND PROCESSED AGRICULTURAL PRODUCTS

Mycotoxins in Peanuts, Pecans, Cottonseed and Aged Hams	John C. Ayres, Professor Food Science Department University of Georgia Athens, Georgia
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SOUTHERN AGRICULTURAL EXPERIMENT STATIONS COLLABORATORS WORKSHOP

March 13-14, 1975

held at

Southern Regional Research Center
Agricultural Research Service
U. S. Department of Agriculture
New Orleans, Louisiana

R E P O R T

The meeting was called to order at 9:00 a.m. on March 13, 1975, by Dr. Mary E. Carter, Director, Southern Regional Research Center.

Dr. Carter welcomed the group to the Center and expressed appreciation on behalf of Dr. Arthur W. Cooper, Administrator for the Southern Region, to the members of the SAES and FDA for their participation in the Workshop.

She then read comments on mycotoxins and food safety prepared by Dr. Harry W. Hays, Staff Scientist, National Program Staff, ARS, USDA, Beltsville, Maryland.

Following her presentation of Dr. Hays' comments, Dr. Carter introduced Dr. William F. Flatt who gave the response and served as General Chairman of the meeting. She also introduced Dr. John W. Sites who served as Discussion Leader for all of the talks presented.

Papers, outlines, or summaries of the talks presented follow.

MYCOTOXINS AND FOOD SAFETY

by

Harry W. Hays, Staff Scientist
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Beltsville, Maryland
(Presented by Mary E. Carter)

On a worldwide basis, natural toxicants and contaminants of natural food products have caused more human injury than any manmade chemical added to food. In Japan, the "yellow rice disease" was associated with a number of molds. In Russia, a severe loss of life was caused by the ingestion of moldy grain. In 1939, horses died in epidemic proportions throughout the Ukraine and the causative agent was identified as a fungal metabolite. In 1960, a major epidemic occurred in Europe when thousands of turkeys died from what was named "Turkey X" disease and which led to the discovery of aflatoxin, a metabolite produced by Aspergillus flavus. This turned out to be the forerunner of a vast amount of research on biochemical processes. The concept that a fungus could produce a metabolite that was a carcinogen immediately suggested to many scientists that mycotoxins could be implicated in the etiology of cancer. This idea was subsequently reinforced by the finding that aflatoxin was not only a carcinogen but that it was the most potent carcinogen known to man.

Liver cancer, like so many other cancers, has a variable distribution throughout the world. There is a high incidence in areas of high humidity and temperature,and these are the two most important factors that favor the production of mold. Therefore, the ingestion of food contaminated with mycotoxins provides a plausible explanation for the high incidence of cancer in those parts of the world where food crops susceptible to fungal attack are stored under primitive conditions.

The difficulty in establishing a cause and effect relationship between mycotoxin and human cancer is the long latent period for the disease to appear. The key question to be answered is whether man is susceptible to the chronic effects of low levels of aflatoxin in our diet. This will require many years of research but it should intensify our efforts to identify other mycotoxins in our agricultural commodities that may have carcinogenic activity. Scientists in ARS have done an outstanding job in dealing with the problems associated with the production of aflatoxin in corn, cottonseed, and peanuts.

In 1965, the late Dr. Marion Parker, who was then Associate Administrator of ARS, spoke before the Institute of Food Technologists and outlined the Department's broad objectives which were:

1. To find practical methods for preventing, or at least minimizing, the growth of mold in agricultural products.

2. To develop rapid methods for detecting aflatoxin.

3. To discover practical methods for removing or destroying toxins that may contaminate agricultural products.

4. To study the metabolic pathways in domestic animals.

The early isolation and determination of the structure of aflatoxin B, led to rapid advancement in developing practical methods of detection, improvement in cultural methods to reduce contamination, and development of physical and chemical methods of decontamination. Although much remains to be done, we must nevertheless ask whether our program needs redirection. Aflatoxin is only one of a number of mycotoxins in our food crops. At present, it is the one most obvious but others are also present. Sterigmatocystin, ochratoxin, and zearalenone have been isolated from various species of Aspergillus, Penicillium and Fusarium molds, and these pose potential health hazards. How long must we continue to develop more sensitive methods or refine physical methods of separation? Detoxification of grain and seed appears to be possible by the use of ammonia but there is uncertainty about the safety of the treated product and possible alteration of nutritional quality. The cost and time spend to get the data required by FDA for safety evaluation must be considered in developing chemical methods of decontamination. Are any other methods available that might effectively reduce the level of contamination and not require FDA approval?

It is my impression that we have become obsessed with aflatoxin and as a result have lost sight of the many facets of mycotoxin research. I hope that you look at the broader aspects of the problem of producing a safe and wholesome food supply.

RESPONSE

by

William P. Flatt, Chairman

Association of Southern Agricultural Experiment Station Directors and Director, Georgia Agricultural Experiment Stations, College of Agriculture, University of Georgia, Athens, Georgia 30602

First, let me say that I appreciate very much the invitation to meet with you here today. I appreciate this opportunity, both for myself and for all the other directors of the Southern Region whom I represent. It is my privilege to be here as their official representative in my capacity as chairman of the Association of Southern Agricultural Experiment Station Directors.

On behalf of my fellow directors, I assure you of our individual and collective interest in the regional research program now underway in this area of Mycotoxins and Food Safety. I want to assure you that we directors are most interested in this research. We recognize its importance to our constituents--the citizens of our respective states. We appreciate its potential for solving some of the most serious problems facing both the producers and processors of some of the most important food crops in our part of the world. I don't have to tell you, for example, how serious a single serious human illness (or death) attributed to aflatoxin contamination of peanut butter would be to the peanut industry in Georgia.

Here I want to say that I am, and other regional directors are, well aware that the mycotoxin program now underway in the region falls far short of the needs as presented in this task force report, "A Program of Research for the Southern Region in Food Safety." We will do everything we can to expand this research as available resources permit.

While I do sincerely appreciate the opportunity to be here, no doubt some of you have been wondering along with me as to why I have been given such a prominent place on the program. In my own wondering about this, I have decided why I was chosen. Granted that it is quite fashionable to have the Administration represented on programs for

meetings such as this, and granted that I qualify as an administrative representative, that really is not why I am here. Or at least not the entire reason. I'm here to represent the non-experts. Without bragging at all, I can assure you that I am eminently qualified as a non-expert on "Mycotoxins and Food Safety."

I do, however, have some thoughts on the subject which I wish to share with you.

Within recent years, Food has become a topic of increasing importance in public affairs. A genuine, serious concern--one not entirely unjustified--among many consumers and their representatives has been an important part of the whole "Protest" or "Complaint" movement best personified by Ralph Nader. This concern already has resulted, as you well know, in stricter requirements for the packaging and labeling of many foods. These stricter requirements apply especially in the area of nutritive content and the presence of food additives.

Another more recent development which has given Food a top spot on the agenda of public concern has been the rapid advance in prices charged for most foods. And here relatively new processing and packaging techniques--more or less demanded by changes in the lifestyles of homemakers--have borne the brunt of the assigned responsibility for the increase in prices.

The very real scarcity of certain foods and the very real possibilities of serious shortages in certain foods also have contributed a lot to this increasing public awareness of foods.

I mention this increased interest in and concern for foods by the consumer public for two reasons.

First, it is quite important to everyone involved in research concerning food safety. It makes what we are doing more important to those people who ultimately will pay for the research. Public awareness of and concern for the need gives us a better chance to be adequately funded to do the job we know needs doing and want to do.

It is quite important to those of us involved in research concerning food safety for a second reason. This new public interest in foods created by rising food costs and food scarcities creates certain new dangers in food utilization. These are dangers which we--along with all others involved in the production and processing of foods--must recognize and prepare to cope with.

First among these dangers is the temptation among food processors to take risky short cuts in accepted and proven procedures taken to ensure

the safety of foods. The purpose of these short cuts would be to reduce processing costs and thereby lower food prices.

Second among these dangers is the temptation among food processors to make substitutions in the ingredients of manufactured foods in the interest of lowering the price. The danger here lies in lack of sufficient information as to how these new ingredients might affect the safety of these foods.

These are temptations which, of course, food processors should resist completely. I am confident that all responsible food processors will resist these temptations. What I am afraid of are those irresponsible food processors who might be willing to run such risks in the interest of making quick profits.

Research scientists, I realize, can do little or nothing to counteract such moves by food processors. It is not the role of scientists to play God or to police an industry. There are ways, however, that research scientists and other educators can help.

First, we must guard against taking any such short cuts ourselves. We must resist strongly the temptation to make recommendations regarding food safety based on incomplete research. And sometimes the pressure to do this might become very great.

Second, scientists can accept the responsibility for seeking, discovering, and verifying new and less time-consuming and less expensive procedures to ensure food safety.

Of course, you and research scientists who are working in other aspects of food safety are aware of these dangers. They are pointed out a number of times in this task force report. In fact, the potential threat of aflatoxin and related mycotoxins in processed food products and research in this area is given first priority in this report. This indicates that scientists are quite willing to fulfill their responsibilities in dealing with these dangers. And I am confident that, as research with mycotoxins in animal feeds and human foods progresses, a lot of the threat of these dangers will be eliminated or at least greatly reduced.

Now, for a few minutes, I should like to discuss another problem which relates to food safety which harbors real danger. This problem is the lack of understanding and knowledge concerning food safety among two important groups of people. These are those people who handle and help prepare commercial foods, and consumers themselves.

Both these dangers recently have been accelerated here in the South. The tremendous expansion in food processing plants and in even greater

increases in reatil outlets for "quick-serve" foods has brought into the food handling labor force a large number of people who have little or no training in personal hygiene and food handling to ensure food safety. Among consumers, the increase in the number of meals eaten or purchased "to go" for eating later, accelerates the dangers of eating contaminated or spoiled foods. Another development among consumers that accelerates and increases the risk is the increase in home production and home preservation of foods.

These dangers also are recognized by research scientists and are noted in this report. I mention them here because I think we educators need to devote more thinking--and more research--to the problem of ignorance regarding food safety among these two groups--food handlers and consumers. Too many of our people are poorly informed, or worse still, misinformed regarding food safety.

This presents special problems for those of us concerned with mycotoxins and food safety. One problem of concern is how to best publicize research with mycotoxins without running the risk of creating aversions to certain important foods that need greater markets. Related to this is how do we prevent publication in the mass media of misinformation about the potential dangers of mycotoxin contamination. We all remember the strawberry scare of several years ago and the more recent erroneous reports concerning corn blight. I don't know the full answer to this problem and would welcome suggestions. I do know that suppression of information about mycotoxin research is not the answer, for it would only make the problem of misinformation worse.

Session I

METHOLOLOGY

IDENTIFICATION OF TOXIGENIC FUNGI

Urban L. Diener, Norman D. Davis, and Gareth Morgan-Jones

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Research in our laboratories has been directed toward the identification of toxigenic fungi and their toxic metabolites. Fungi have been isolated from three broad sources: mixed feeds (thermophilics), moldy human foods, and bermudagrass hay. Three experimental bioassays were utilized: primary being brine shrimp, secondary the chick embryo, and finally the rat bioassay by intraperitoneal injection or by oral administration.

Fungi were grown on a medium developed by Kirksey and Cole (4) composed of 50 g shredded wheat and 100 ml of nutrient solution in each 1-liter flask. After sterilization and inoculation, flasks were incubated at 26 C for 14-21 days, sometimes followed by 14 days at 11 C as described by Christensen et al., (1). Cultures were extracted with chloroform, chloroform-ethanol, or chloroform-methanol (80-20) in a Waring blendor, filtered, and the combined filtrates concentrated to about 50 ml under an airstream at room temperature. The extract was divided into two equal portions for the brine shrimp and chick embryo bioassays.

The brine shrimp bioassay was essentially that described by Harwig and Scott (3) except that the duration of the test was 4 hr. The 25 ml portion of the extract was completely dried under airstream and vacuum oven and taken up in 10 ml of 95% ethanol. Two drops (.04 ml) of this extract were placed in the well of a Pyrex 7220 chemical spotting plate, which was filled up with brine shrimp-salt water solution to give approximately 1 ml final volume (final ethanol concentration of 4%). Controls consisted of the same mixture with the extract being from uninoculated substrate. Plates were incubated at 27-29 C in high relative humidity.

The chick embryo bioassay methods were those described by Verrett et al. (5) and by the AOAC for aflatoxin bioassay (1). The portion of extract for this bioassay was transferred to a 50-ml flask containing 1.5 ml of peanut oil. The flask was plugged with cotton and heated on a boiling water bath permitting the chloroform-ethanol fumes to sterilize the flask and peanut oil. After most of the solvent had evaporated, the remaining solvent was removed in a vacuum oven at 50 C for 24 hr. Flask contents were kept sterile by the cotton plug held in place by a piece of cheesecloth and a rubber band. Locally obtained fertile chicken eggs were incubated 5 days until the blood vessels of the embryo were clearly visible on candling. Eggs were surface sterilized above the air sac with 5% iodine in 70% ethanol prior to the puncturing of the shell with a sterile 22 G needle in a rubber stopper before injection. Sterile disposable syringes were used to inject 0.1 ml of fungal extract-peanut oil suspension into each egg. For each extract, five eggs were inoculated in the air sac and another five in the yolk. Eggs were incubated at 40 C and 84-86% RH in a Leahy model 624 incubator. Eggs were turned three times daily and data on embryo mortality were recorded after 4 days. Sac and yolk mortality were combined. The experiment was repeated one or more times.

The rat bioassay was conducted with fungal extract-peanut oil or corn oil suspensions prepared with the contents of a second flask by the method described for the previous bioassays. Charles River (CD), 21-day old weanling rats (4-6/treatment) were given water but no food for 24 hr prior to treatment. Each rat was injected ip with approximately 0.4 ml of fungal extract-oil suspension. Following injection rats were given food and water ad libidum. Observations were made for several hours after injection, daily, and upon autopsy following the 7th day. Rats were weighed before injection and again at the end of the experiment. Weight gain or loss relative to the control group, which were injected with extract-oil suspensions from uninoculated medium, was calculated for each group of treated rats.

For oral administration, the extract from 1-3 flasks (depending on the number of rats per treatment) and 6 ml of peanut or corn oil were transferred to a 50-ml or 125-ml Erlenmeyer flask, placed on a water bath to remove most of the solvents and to sterilize the flask and oil, and then placed in a vacuum oven for 18-24 hr. Extract-oil suspensions were administered with an intubation needle with a ball tip. The ball tip was dipped in oil for smooth entrance into the esophagus. Each rat was fed 1/2 of the extract (0.5-1 ml) on both day 1 and 2 by intubation. Control rats were fed extract-oil suspensions from uninoculated shredded wheat-nutrient amended medium. Experiments were conducted for 14 days during which rats had rat chow and water ad libidum. Rats were weighed initially, at demise or at the end of the experiment. Upon death and termination of the experiment, treated and control rats were autopsied. Data were recorded on hemorrhaging, stomach ulcers, abnormalities of liver, spleen, heart, kidneys, etc. in comparison to control rats. Date of death and notes on physical appearance, nervous reactions, etc. were recorded when noted. Mean average weight gain or loss in comparison to control group was also determined.

When more than one flask of fungus-shredded wheat residue remained after extraction, it was combined for each treatment, dried in an oven at 45-50 C for 24 hr, and ground in a food chopper. This ground residue was mixed 50:50 with ground rat chow using corn or peanut oil as a binder and fed ad libidum. Food containers were filled with adequate feed daily and were cleaned every 3rd day to avoid mold in humid weather.

Relative toxicity of extract or residue to rats was determined from data on: (A) Mortality of rats in the treatment groups, (B) Rate of weight gains of treated versus control group of rats, (C) Pathological observations (by DVM) and effects on organs, and (D) Weight gains exceeding controls by 20% or more were noted as indications of other biological activity.

RESULTS

Thermophilics were grown at 50 C, but otherwise the procedures were the same in extracting and bioassaying fungal cultures. These data being presented here will appear in the April issue of Applied Microbiology. Fungi were obtained both by isolation from feeds and local materials as well as from Emory Simmons, a Canadian mycologist.

Slides: Table 1 and 2 indicate the fungi screened and their source. Bioassays used were brine shrimp, chick embryo, and rat (ip). Tables 3 and 4 present the data from the bioassays.

Fungi were isolated from moldy foods obtained from a local supermarket.
Slides: Tables 1, 2, and 3 show the results of testing a large number of fungi by the brine shrimp bioassay. Those fungi giving any data showing toxicity were also tested by the chick embryo bioassay. Tables 4 and 5 present the results of this bioassay. Table 6 summarizes these data by pointing out the principal mycotoxins produced by some of these fungi and also those not known to produce toxic metabolites or where the toxic factors have not been identified.

Fungi have been isolated from moldy bermudagrass periodically since 1971, particularly whenever hay has been suspect of being a source of toxicity to cattle. In 1973 and 1974, several farmers had problems notably in Autauga county just north of Montgomery.
Slides: Table 1 shows the fungi isolated primarily in 1973 and identified by Gareth Morgan-Jones. Slides 2-6 are from some drawings by the mycologist. Both Pithomyces and Periconia (several species) have been found but do not appear to be involved in tremors. Table 2 and 3 reveal the toxicity of the several fungi to both brine shrimp and chicken embryos.

In September of 1974, we had an outbreak of bermudagrass tremors or staggers at the Plant Breeding Unit of the Agronomy Dept. of the Alabama Agricultural Experiment Station near Tallassee, AL about 35 miles from Auburn. The predominant fungus present was none of those previously reported. This had been confirmed by some preliminary studies of exploratory nature, in which eight of them were tested by growing them on a half a bale of hay. The feeding tests on a known susceptible calf had no effect. However, the fungus finally isolated, although identified, has not yet been proved to be the toxin producer or even to be the obvious fungus observed in the field. We are actively pursuing investigations in both directions.
Slides: 1 - Introduces Norman D. Davis and Gareth Morgan-Jones in the pasture, where the toxic bermudagrass was present.
2 - Introduces one of the cast and the cameraman of the movie, which is to follow that clearly illustrates the effects of the toxin in two cattle in the field that were put back on the pasture for several days. The second part of the film is of a calf that got the tremors after eating between a sixth and a quarter of a bale of hay from the orchard.
Movie: 16 mm - 8 minute movie illustrating the signs and symptoms of bermudagrass tremors in cattle.

Literature Cited

1. Association of Official Analytical Chemists. 1970. Official methods of analysis, 11th ed., sec. 26.057-26.061. Washington, D.C.

2. Christensen, C. M., G. H. Nelson, C. J. Mirocha, and Fern Bates. 1968. Toxicity to experimental animals of 943 isolates of fungi. Cancer Res. 28:2293-2295.

3. Harwig, J., and P. M. Scott. 1971. Brine shrimp (Artemia salina L.) larvae as a screening system for fungal toxins. Appl. Microbiol. 21:1011-1016.

4. Kirksey, J. W., and R. J. Cole. 1974. Screening for toxin-producing fungi. Mycopathol. Mycol. Appl. 54:291-296.

5. Verrett, M. J., J. Marliac, and J. McLaughlin, Jr. 1964. Use of the chicken embryo in the assay of aflatoxin toxicity. J. Ass. Offic. Anal. Chem. 47:1003-1006.

COMMENTS ON MYCOTOXIN STUDIES AT LSU

by

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We became involved in mycotoxin studies some seven years or more ago. Domiciano Broce of Panama was involved in studies under Dr. Robert Grodner of the Food Science and Technology Department. He wrote his dissertation on his studies on ochratoxin.

The Food Science Department reported problems with their flour through Mr. Joe Killebrew, Assistant Food Production Manager, who noticed black splotches on pie shells which were held in a dough retarder before baking. After the shells were baked, the black splotches appeared. Mr. Killebrew thought the trouble could be from a fungus. Samples were taken under aseptic conditions, and Broce and I extracted the hard wheat flour samples. Aflatoxins B_1, B_2, G_1, G_2 were detected by T.L.C. The cultures made from the flour contained Aspergillus flavus and some other common molds. Flour samples were treated with heat (autoclaving) and by irradiation with Cobalt 60 at very high levels. The flour from the treated samples held no viable spores. We then irradiated the cultures and extracted them. They still produced aflatoxins. A paper on this study was presented at the American Nuclear Society Convention in Toronto, Canada, the next year.

Since then we have made several studies including the work on Coastal Bermuda Grass. From this study two species of Aspergillus and one Claviceps sp. were found which produced tremorgenic substances. The work is still in progress.

A routine screening is made of samples sent by farmers for all known mycotoxins. Several field specimens of hay and corn have produced aflatoxins and sterigmatocystin and patulin.

Dr. Dale Newsom's group has recently extracted a substance from Coastal Bermuda Grass which is now being tested. Our work at LSU is on a collaborative basis intradepartmentally.

Data from this study was sent to Dr. Porter and Dr. Bacon for inclusion in a paper.

DETECTION OF FUNGI IN FEEDS AND FORAGES AS RELATED TO POOR PERFORMANCE IN LIVESTOCK AND POULTRY

by

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The measurement of fungal growth in agricultural products is essential for determing the extent of contamination and predicting if these products may cause problems if ingested. Methods currently used do not distinguish between growth and reproductive structures. Those of us who are concerned with the problems of mycotoxins realize that these compounds are produced as a consequence of fungal growth at some point in time and have very little to do with reproductive structures. Results from the usual methods of estimating fungi (the colony count and its many modifications) often mean that for any given sample there are numbers of reproductive structures (sexual and/or asexual). These results would represent the ability of a fungus to produce many spores and have no relationship to growth on that substrate. Of course this method does allow one to identify the fungus and thus determine viability. The method does not distinguish between soil and air borne contaminants. Exceptions do exist, but I believe that in the final analysis these exceptions would be cases where the particular substrate is completely contaminated with one or maybe two fungi. Then too, there are the cases where some fungi simply do not sporulate or those that do, cannot be grown out on conventional laboratory media used in the colony count procedure. An example of this latter case would be some of the systemic phytopathogens that we are currently studying at the Richard B. Russell, Agricultural Research Center.

The two methods that I want to describe would not differentiate between living and dead fungi and they do not identify the fungi. One method would conveniently indicate the amount of mycelium while the other implies toxicity. A combination of the colony count procedure with one or both of these methods may lead to a true accessment of fungal contamination.

The first method is a relatively rapid colorimetric procedure basing the amount of mycelial growth on the quantity of glucosamine liberated during the alkaline hydrolysis of chitin. The second method is not necessarily a means of estimating fungal growth, our present information indicate that it is a "fungal fingerprint" of toxic fescue and Bermuda grass. In addition to discussing these methods, I will present data obtained from them to demonstrate their usefulness and which will describe our work at the Athens area. Experimental details of these methods may be obtained from the references.

The method used to determine the mycelial content in natural products is based on the assumption that if a compound is contained in mycelia at a constant rate and not present in that natural product, the amount of mycelia can be determined from the content of the compound in that natural product. Of the many constituents of the mycelium, chitin is a component of most fungal cell walls and is unique in being absent from most higher plant and animal cells. The method is modified from Rides and Drysdale (1972).

A summary of the procedure (Table 1) indicates that in addition to estimating mycelium the organic solvent used in extracting plant pigments may be saved and analyzed for mycotoxins. Briefly the method involves the hydrolysis of chitin in the sample with concentrated KOH at 130 C for 1 hr. The hydrolysis causes a depolymerization and deacetylation of the chitin to produce a group of compounds called chitosan. The chitosan is deaminated with nitrous acid to yield the aldehyde, 2,5-anhydromannose. The aldehyde is determined colorimetrically with a 3-methyl-2-benzothiazolone hydrazone according to Tsuji et al. (1969). Glucosamine is used as a standard and the resulting blue color is read at 650 nm against a reagent blank. The colorimetric procedure is sensitive (0.1µg) and specific, however, it will react with galactosamine.

This method is also based on the assumption that during deamination the amount of chitosan converted to the aldehyde is reasonable constant--it is not total. However, replication and the sensitivity of the procedure circumvent this aspect. I suppose samples known to consist of ingredients containing chitin from other sources could be analyzed with appropriate controls. I have not tested samples

of this type. Generally there are two ways of expressing results: (1) unit of fungus/unit of substrate and (2) unit of glucosamine/unit of substrate.

This latter expression of results is better suited for routine analysis of samples where one is interested primarily in determing contamination. This choice of expressing results is shown in Table 2. The data represent a comparison of the colony count with chitin content. The data from the colony count represent averages from 10 laboratory media. Note that the amount of fungi is expressed as milligram glucosamine per gram of sample. There are some similarities between colony count and glucosamine content, e.g. field damaged soybeans and toxic poultry feed. There are differences, in both undamaged soybeans and poultry feed the glucosamine content is zero while the colony count for these indicate that fungi are present. What this means is that in damaged soybeans and toxic poultry feed there was fungal growth as the chitin procedure does not measure fungal spores since these structures contain little chitin. This illustrates that the chitin method differentiates between fungal growth and fungal spores.

The fescue samples illustrate another point (Table 2). The colony count procedure shows no significant difference between toxic fescue and nontoxic fescue whereas there are differences in their glucosamine values. While the source of fescue differed geographically, the densities of each species were similar. The higher glucosamine content in the toxic fescue over nontoxic fescue could be interpreted as growth of a fungus as indicated above. I shall analyze these data later and show that this is indeed the case. The fungus, Balansia epichloë (weese) responsible for this growth could be detected in the colony count procedure as it is a systemic phytopathogen.

The alternative means of expressing results, i.e. mg fungus/g substrate, is more suited for laboratory studies. As I said earlier this method is based on the assumption that the chitin content is constant. In order to equate mg of glucosamine to mg of a species you must know the value of this constant since the content of chitin may vary with age, cultural conditions and is species dependent. These variables must be evaluated for each

fungus and a glucosamine value (chitin content) determined for cultural conditions. This value may then be used to determine the amount of fungus per unit of substrate. For example, this method will allow one to quantitate growth and mycotoxin production on a substrate under environmental influences as the following experiments will demonstrate.

In Figure 1 are results from an experiment used to obtain the glucosamine value of Aspergillus ochraceus grown on a yeast-sucrose medium as stationary cultures. This fungus produces ochratoxin A and penicillic acid. From this experiment a glucosamine value of 35.25 µg/mg of fungus (dry wt) was obtained; there was a linear relationship between growth (dry wt) and glucosamine content. The production of ochratoxin A on poultry feed was studied using the glucosamine value of 35.25 µg/mg. The results of that study are presented in Figure 2. The pattern of ochratoxin A production and growth on poultry feed is similar to that in surface and submerged culture of this fungus on liquid media. However, the maximum amount of toxin produced is higher on poultry feed than on liquid substrates. Now, are the conditions necessary for maximum growth on poultry feed also optimal for maximum mycotoxin production? An answer to this question is shown in Tables 3 and 4. In Table 3 an a_w of 0.95 and a temperature of 30 C is required for maximum production of ochratoxin A and growth. However, penicillic acid requires a temperature of 15 C and an a_w of 0.99 and 20 C at an a_w of 0.90; growth is not optimal at these conditions. Thus, penicillic acid production is more dependent on lower temperatures and conditions less favorable for optimum growth. The requirements for maximum ochratoxin A production are similar to those for growth. That these requirements are the same for all natural products are unknown.

Does maximum growth limit mycotoxin production on different agricultural products? I am not implying that maximum growth is necessary for mycotoxin production. I do mean that there is a critical amount of growth necessary for some mycotoxin formation. The data in Tables 5 and 6 indicate that under similar condition of temperature (25°C) and moisture (a_w 0.95) growth is not a limiting factor for ochratoxin A production. You see, many of these substrates have similar amounts of growth but significantly lower amounts of ochratoxin A produced, e. g., poultry feed and oats, pecans and

sunflower. If the initial assumption is true, these data suggest that growth is not a limiting factor for the production of this mycotoxin and imply that other factors (possibly inherent to the substrate) are responsible for the varying amounts of toxin produced. We are examining that implication.

The other method that I want to describe is one developed from a series of studies on the etiologies of the fescue foot and Bermuda grass tremors syndromes. To reemphasize, this method is not a means of quantitating fungi and it is best described as an indicator of fungi on grasses. Much of this work was done by Drs. J.K. Porter and J. D. Robbins at the Richard Russell Research Center. Dr. Porter, the chemist of the group, determined the chemical structures that form the basis of this precedure (Porter et al. 1975).

Before I discuss this method, I believe that a brief review of the signs of fescue foot and Bermuda grass tremors would help those who are unfamiliar with these two syndromes. In Table 7 are listed the clinical signs of fescue toxicity in animals. Cattle in some herds may show from one to several of these signs, i.e., these signs are not listed in order of occurrence. In Figure 3 is a picture of a cow from a Georgia toxic fescue pasture showing some of these signs: loss of weight, rough-hair coat, diarrhea, and lameness. In the following Figure 4 is a closeup of that cow showing that in addition to these other signs, she also has lost the end of her tail.

Bermuda grass tremors cannot be adequately described by still photographs. The syndrome occurs in cattle grazing common and coastal Bermuda grass (Cynodon dactylon) and is characterized by a general nervousness in cattle which varies from a slight twitching of the muscles in the shoulders and flank regions, to an inability to stand or walk because of posterior paralysis.

The clinical picture suggest that ergot or related alkaloids are involved with fescue foot (gangrenous ergotism) and Bermuda grass tremors (convulsive ergotism). The compound involved with the fescue foot is considered to be a vasoconstrictor. While studying fungi associated with grasses of these two syndromes, two clavicipitaceous fungi were isolated: Claviceps from toxic Bermuda grass and Balansia from toxic fescue and Bermuda grasses.

Claviceps, a noted producer of biologically active alkaloids, parasitizes the ovary of many grasses and sedges. Balansia differs from Claviceps in the degree of parasitism, it is systemic, living in various grasses, growing and invading new tissue as the grass grows. In Figure 5 the fruiting bodies of Balansia epichloë are shown on smut grass. This species of Balansia was also found on fescue, love grass (Eragrostis hirsuta) and panicum (Panicum anceps). Cattle graze all these grasses. Infected clones of fescue do not produce seed, and are dwarfed and fasciated presumably due to shortening of the internodes. The black ergot-like pseudomorphis ascostramata of the fungus are seasonal dependent and ephemeral (Figure 6). The white asexual stage of this fungus, the Ephelis state, is shown in Figure 7. In addition to this species of Balansia, B. henningsiana was also isolated from toxic fescue pastures. The species of Balansia from toxic Bermuda grass was not identified. Since Balansia is so closely related to Claviceps we are currently determining if it might be the cause of Fescue foot syndrome and Bermuda grass tremors. Balansia epichloë is toxic to the chicken embryo and produce at least 3 toxic indole compounds in vitro (Table 8). One of these compounds, A-2, is chloroform soluble and is extractable with tartaric acid from chloroform. The structures of these compounds are not known.

Chloroform extracts of toxic Bermuda grass and two isolates of Claviceps from toxic Bermuda grass showed a blue fluorescent band visible at 366 nm (Figure 8). We considered this band significant enough to screen for it in other samples and Balansia. In Figure 9 are chloroform extracts of toxic Bermuda grass, Balansia and nontoxic Bermuda grass. The blue fluorescent band is absent in nontoxic Bermuda grass but present in toxic Bermuda grass and Balansia. Because Balansia was also isolated from toxic fescue would this grass also contain this band? In Table 9 we see that this blue fluorescent band was also present on toxic fescue but absent from nontoxic fescue samples. The band is also present on smut grass, the source of the isolate of B. epichloë.

The identity of this compound was aided greatly by achieving the vitro culture of Balansia epichloë on a synthetic medium. The mycelium from the fungus cultured on this medium contained enough of the compound for Dr. Porter to do the chemistry.

Gas-liquid chromatography separated this one blue bank into two compounds. By spectroscopic analyses, these two compounds were identified as ergosta-4,6,8 (14), 22-tetraen-3-one (1) and ergosta-4,6,8 (14)-trien-3-one (11) (Fig. 10). The uv absorption of these two compounds is presented in Fig. 11. While there is no quantitative value, compound I was found in a higher concentration than compound II. For the sake of simplicity these indicator compounds will be referred to as the "ergostaenes" I or II. Ergostaene I was synthesized from ergosterol (Figure 12) and this synthetic product used as the standard during our screening of toxic grasses. The infrared spectra of the synthetic and the natural product are presented in Figure 13.

The toxicity of the ergostaenes to cattle is unknown. The ergostaenes are not toxic to the chicken embryo. Toxicity studies of these compounds must be done (not necessarily by our group). Additionally, the effect of these compounds on the grasses should also be studied.

The procedure used to isolate the ergostaenes from forages is presented in Figure 14. The isolation of the compounds from fungi may be simplified by omitting the column chromatography steps. If you are interested in obtaining the ergostaenes from fungi you must use the mycelium, very little of the compounds is produced in the liquid medium that we used. Routine analyses of grass samples indicate that sampling time and methodology are critical. This precaution developed from analyses of known toxic bales of grass and toxic pastures. The variability of toxicity signs of both syndromes in herds coincide with the variability (concentration?) of the ergostaenes in a pasture. Ergostaenes I varied among samples randomly selected from bales of hay known to be toxic and also from the same bale. This suggest that the sampling procedures is very critical. We have no idea as to the appearance or disappearance of the ergostaenes in toxic pastures. Dr. Robbins is studying this aspect in two varieties of fescue and he also is concerned with the concentration of these compounds in grasses.

References

1. Porter, J. K. Bacon, C. W., and Robbins, J. D. 1975. A field indicator in plants associated with ergot-type toxicities in cattle. J. Agr. Food Chem. (In press)

2. Ride, J. P., and Drysdale, R. B. 1972. A rapid method for the chemical estimation of filamentous fungi in plant tissue. Physiol. Plant Pathol. 2:7-15.

3. Tsuji, A., Kinoshita, T., and Hoshino, M. 1969. Analytical chemical studies on amino sugars. II. Determination of hexosamines using 3-methyl-2-benzothiazolone hydraxone hydrochloride Chem. Pharm. Bull. 17:1505-1510.

TABLE 1

SUMMARY OF METHOD
(J. P. RIDE AND R. B. DRYSDALE)*

1. SAMPLE IS HOMOGENIZED IN A SOLVENT.
   A. SOLVENT MAY BE USED FOR MYCOTOXIN ANALYSIS.
   B. SAMPLE RESIDUE IS WASHED WITH H_2O.
2. SAMPLE RESIDUE (CHITIN) IS CONVERTED TO CHITOSAN BY ALKALINE HYDROLYSIS (1 HR AT 130°C).
3. CHITOSAN IS DEAMINATED (ALDEHYDE).
4. COLORIMETRICALLY DETERMINED AT 650 NM.

*PHYSIOLOGICAL PLANT PATHOLOGY. 1972. 2:7-15.

TABLE 2

COMPARISON OF METHODS FOR

ESTIMATING FUNGI

SAMPLE	GLUCOSAMINE MG/G	COLONY COUNTS COLONIES/G
DAMAGED SOYBEANS	81.6	3.6×10^5
UNDAMAGED SOYBEANS	0.0	1.5×10^2
TOXIC POULTRY FEED	39.0	2.1×10^6
POULTRY FEED	0.0	1.1×10^3
TOXIC FESCUE	75.0	4.2×10^6
NON-TOXIC FESCUE	3.6	3.1×10^6

TABLE 3

GROWTH AND PRODUCTION OF OCHRATOXIN A ON POULTRY FEED BY *ASPERGILLUS OCHRACEUS*

a_w	OCHRATOXIN A AND TOTAL GROWTH*		
	15°C	20°C	30°C
0.9011	0 9.02	40 16.78	122 25.15
0.9532	31 15.00	210 26.31	320 34.51
0.9966	84 15.33	149 25.31	299 30.52

*Ochratoxin A expressed as µg/g feed (dry wt.)
Total growth expressed as mg/g feed (dry wt.)

TABLE 4

GROWTH AND PRODUCTION OF PENICILLIC ACID ON POULTRY FEED BY *ASPERGILLUS OCHRACEUS*

a_w	PENICILLIC ACID AND TOTAL GROWTH*		
	15°C	20°C	30°C
0.9011	183 9.02	478 16.78	289 25.15
0.9532	284 15.00	300 26.31	115 34.51
0.9966	400 15.33	279 25.31	39 30.52

*Penicillic acid expressed as µg/g feed (dry wt)
Total growth expressed as mg/g feed (dry wt)

TABLE 5

GROWTH AND OCHRATOXIN A PRODUCTION ON FEEDSTUFFS
BY ASPERGILLUS OCHRACEUS

	GLUCOSAMINE CONTENT		
SAMPLE	BLANK VALUES MG/G SAMPLE	GROWTH MG FUNGUS/G SAMPLE	OCHRATOXIN A MG/G
BARLEY	0.00	26.28	0.68
WHEAT	0.00	26.42	0.68
RYE	0.00	35.42	1.10
OATS	0.00	24.28	0.60
CORN	0.00	24.00	0.27
RICE	0.00	21.28	0.71

TABLE 6

GROWTH AND OCHRATOXIN A PRODUCTION ON FEEDSTUFFS BY *ASPERGILLUS OCHRACEUS*

	GLUCOSAMINE CONTENT		
	BLANK VALUES MG/G SAMPLE	GROWTH MG FUNGUS/G SAMPLE	OCHRATOXIN A MG/G
SOYBEAN	0.00	22.43	0.55
PECAN	0.00	19.14	0.70
PEANUT	0.00	32.86	0.72
SUNFLOWER	0.00	19.71	0.29
ALFALFA	0.00	11.57	0.00
POULTRY FEED	0.00	24.43	3.56

TABLE 7

CLINICAL SIGNS OF
GANGRENOUS ERGOTISM OR
FESCUE TOXICITY IN CATTLE

Loss of weight, emaciation
Rough hair coat
Diarrhea
Tendency to lie in cool places
Increased respiratory rate
Loss of end of tail
Discoloration of tips of ears
Lameness
Loss of rear foot, gangrene

TABLE 8

ULTRAVIOLET ABSORPTION SPECTRA OF ALKALOIDS FROM BALANSIA EPICHLOË

COMPOUND	λ CH_3OH MAX			
A-1	221	272	279	288
A-2	221	275	282	291
B	220	273	280	289
AGROCLAVINE	224	275	283	293
INDOLE	218	271	278	287

Table 9. Tlc results of $CHCl_3$ extracts of Ky 31 fescue, Kenhy, and Smut grass on silica gel GF 254; solvent system $CHCl_3$ - $(CH_3)_2CO$ 93:7, v/v).

No.*	Sample Var. (Yr.)	Toxic	Nontoxic	Fluorescent Indicator
1.	Ky 31 (1973)	+	-	+
2.	Smut grass (1972)	+	-	+
3.	Smut grass (1973)	+	-	+
4.	Kenhy (1973)	+	-	+
5.	Ky 31 (1973)	+	-	+
6.	Kenhy, Rep I (1974)	-	+	-
7.	Kenhy, Rep III (1974)	-	+	-
8.	Ky 31, Rep I (1974)	-	+	-
9.	Ky 31, Rep III (1974)	-	+	-
10.	Kenhy Fd (1974)	-	+	-
11.	Ky 31 (1974)	-	+	-

*Samples 1-3, Newton Co. Ga.; Samples 4-11, Lexington, Kentucky.

Figure 1. Glucosamine control, Dry weight, and ochratoxin A production by Aspergillus ochraceus in stationary culture on a 30% sucrose and 1% yeast extract medium; glucosamine value, 35.25 μg/mg fungus, dry weight.

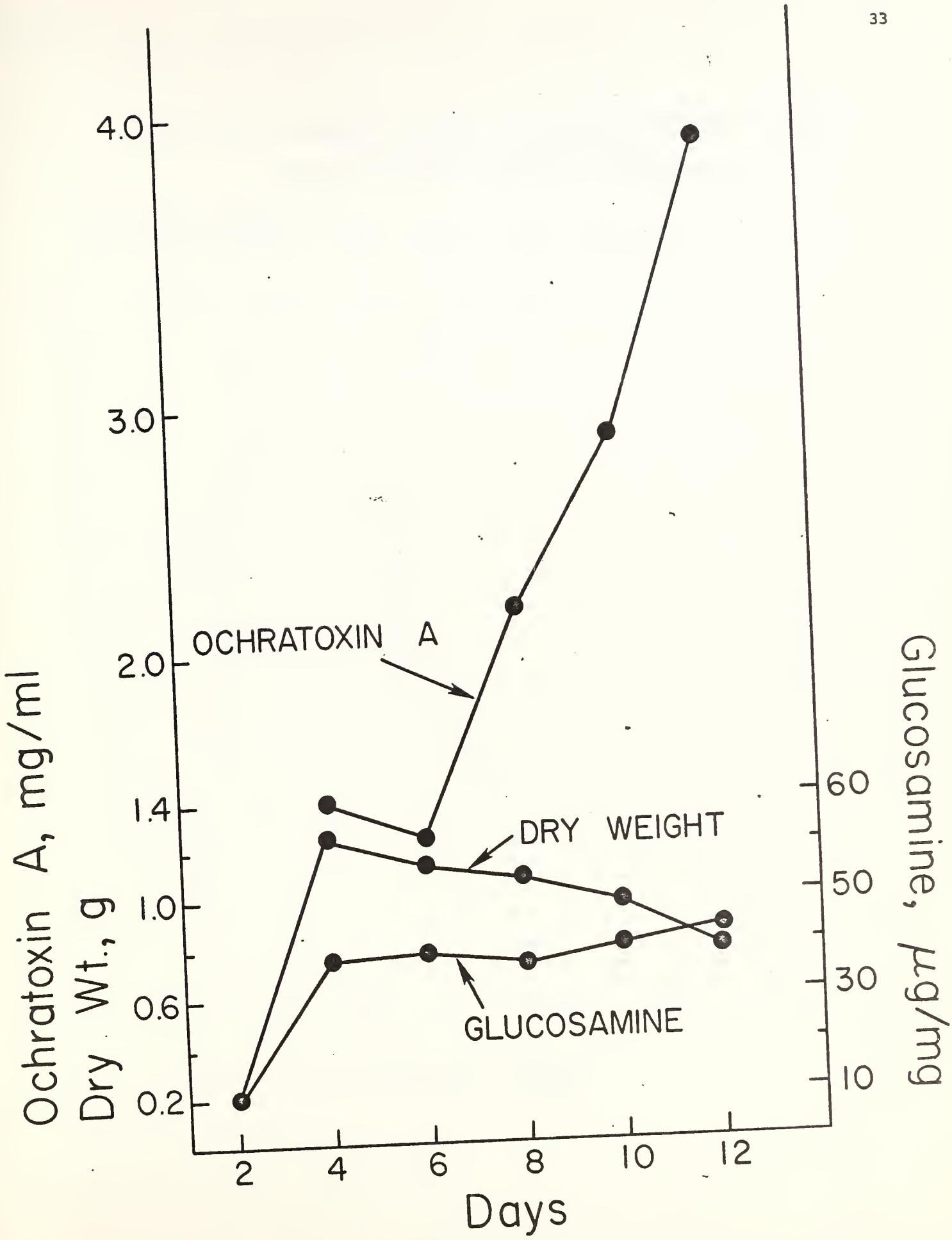

4.0
3.0
2.0
1.4
1.0
0.6
0.2
OCHRATOXIN A
DRY WEIGHT
GLUCOSAMINE
Ochratoxin A, mg/ml
Dry Wt., g
60
50
30
10
Glucosamine, μg/mg
2
4
6
8
10
12
Days

Figure 2. Growth and ochratoxin A production on poultry feed; growth was measured by using the value 35.25 μg/mg.

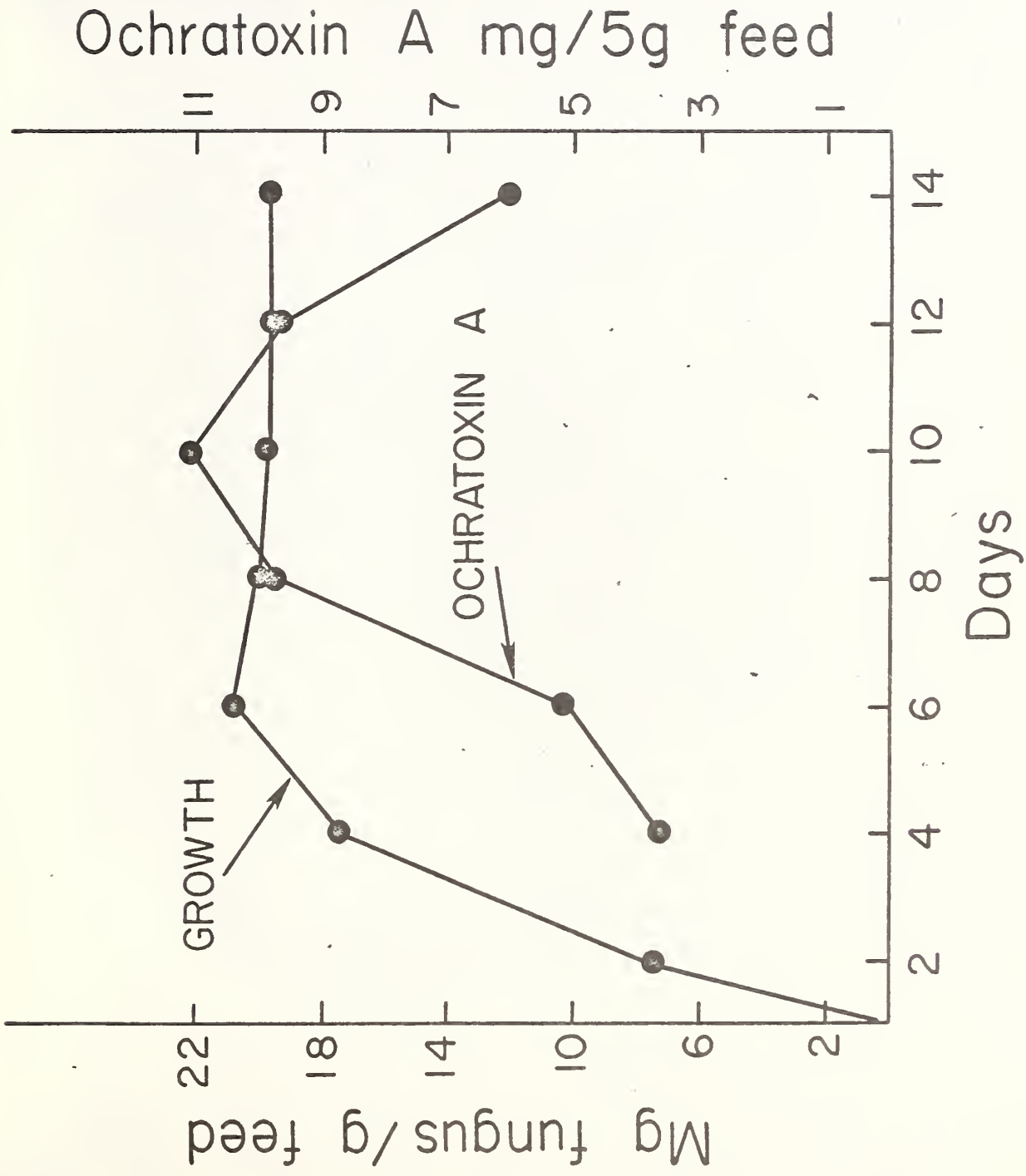

Ochratoxin A mg/5g feed
11
9
7
5
3
1
Mg fungus/g feed
22
18
14
10
6
2
GROWTH
OCHRATOXIN A
2
4
6
8
10
12
14
Days

Figure 3. A cow from a Georgia toxic fescue pasture showing several signs of fescue foot syndrome.

Figure 4. A closeup of a cow's tail showing that she has lost the end of her tail.

Figure 5. *Balansia epichloë* sporulating on smut grass (*Sporobolus poiretii*) in a toxic fescue pasture.

Figure 6. The black ergot-like pseudomorphic ascostromata of B. epichloe on the adaxial leaf surface of the grass.

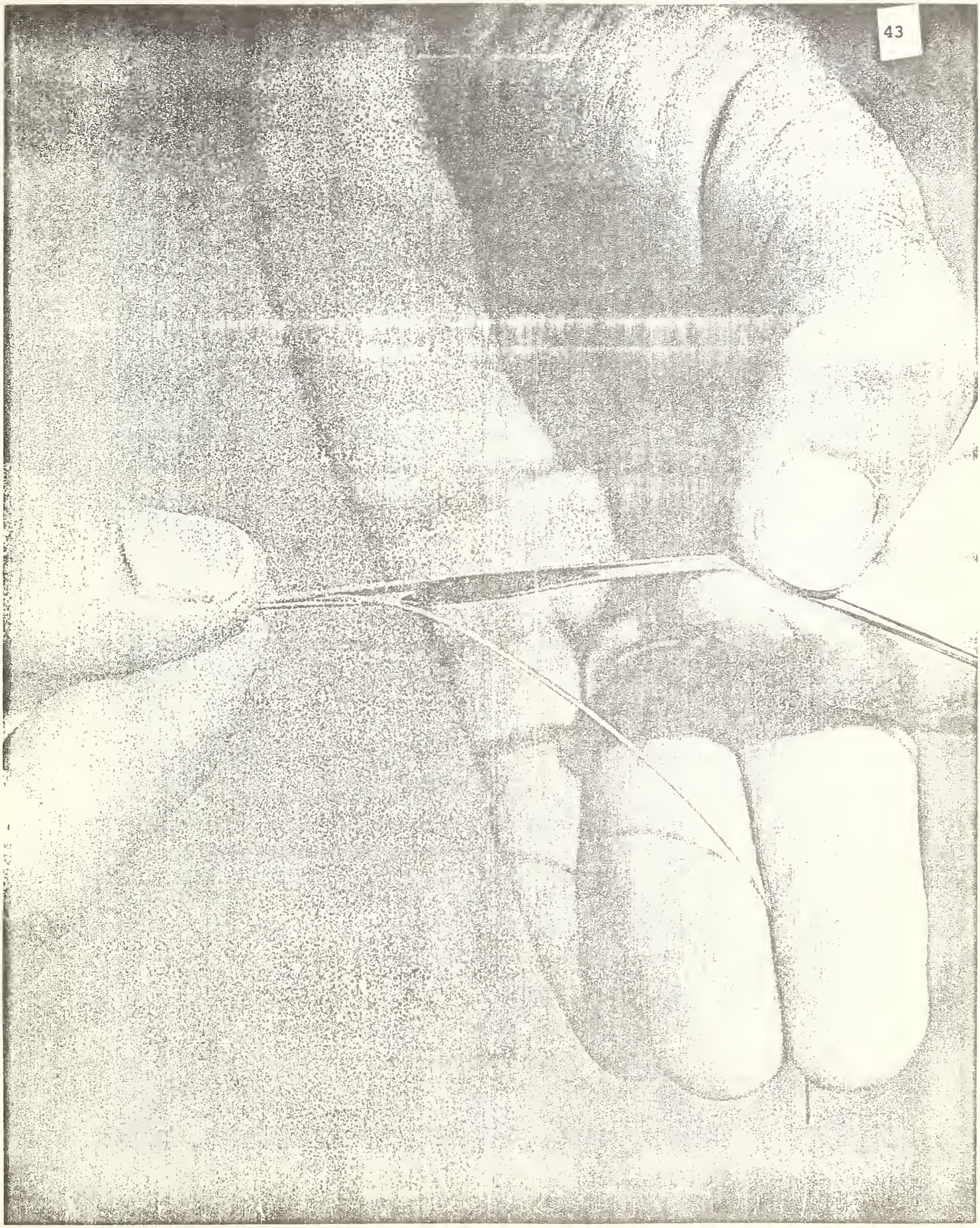


Figure 7. The *Ephelis* state of *B. epichloë*.

Figure 8. Tlc of isolated fluorescent fraction on silica gel GF 254. Solvent system: $CHCL_3$-$(CH_3)_2CO$ (93:7, v/v): (1) Claviceps 174; (2) Claviceps 178 (3) Claviceps 174 (4) Toxic Cynodon dactylon, Louisiana, 1971.

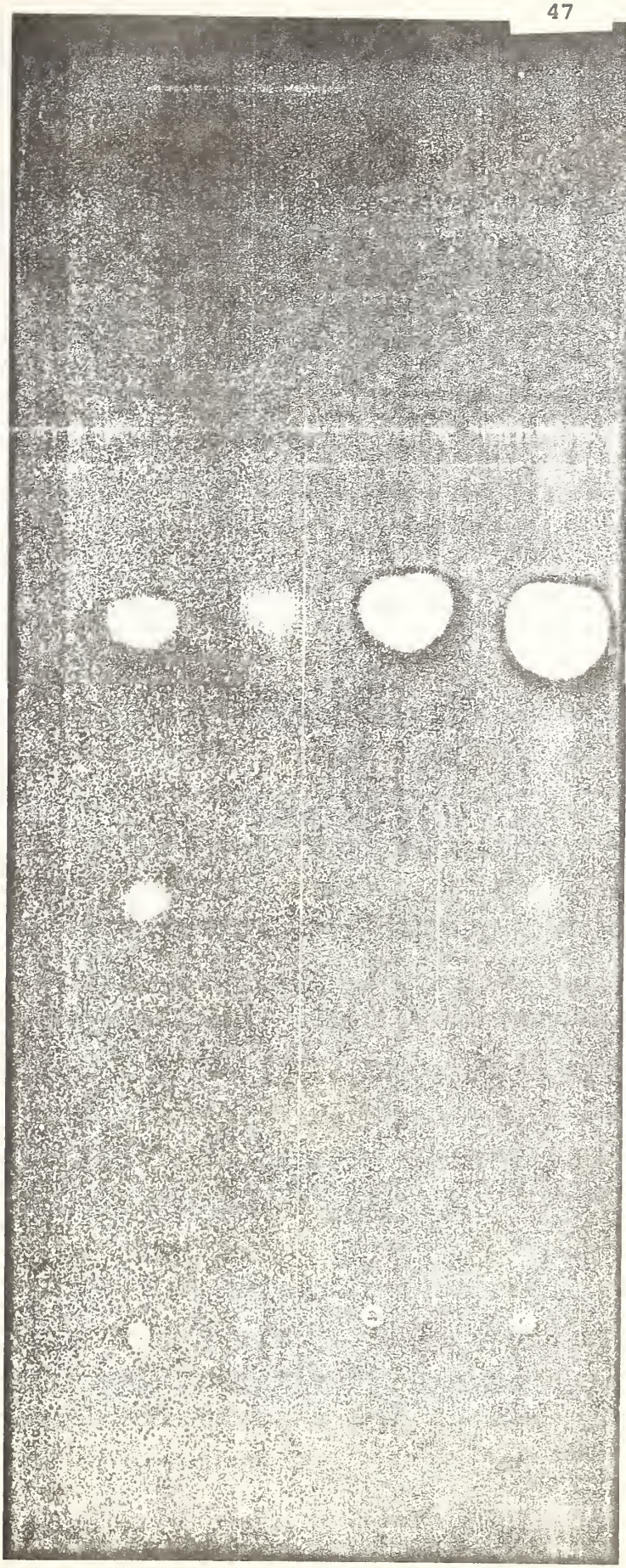


Figure 9. Tlc of $CHCL_3$ extract of Cynodon dactylon and Balansia epichloë on silica gel GF 254. Solvent system: $CHCL_3$-$(CH_3)_2CO$ (93:7, v/v); (1) nontoxic Louisiana, 1971; (2) toxic Louisiana, 1971; (3) toxic Mississippi, 1972; (4) toxic Texas, 1972; (5) toxic Louisiana, 1973; (6) toxic Alabama, 1974; (7) nontoxic Georgia, 1972; (8) B. epichloë isolate from Sporobolus poiretii; (9) B. epichloë isolate added to nontoxic Georgia, 1972.

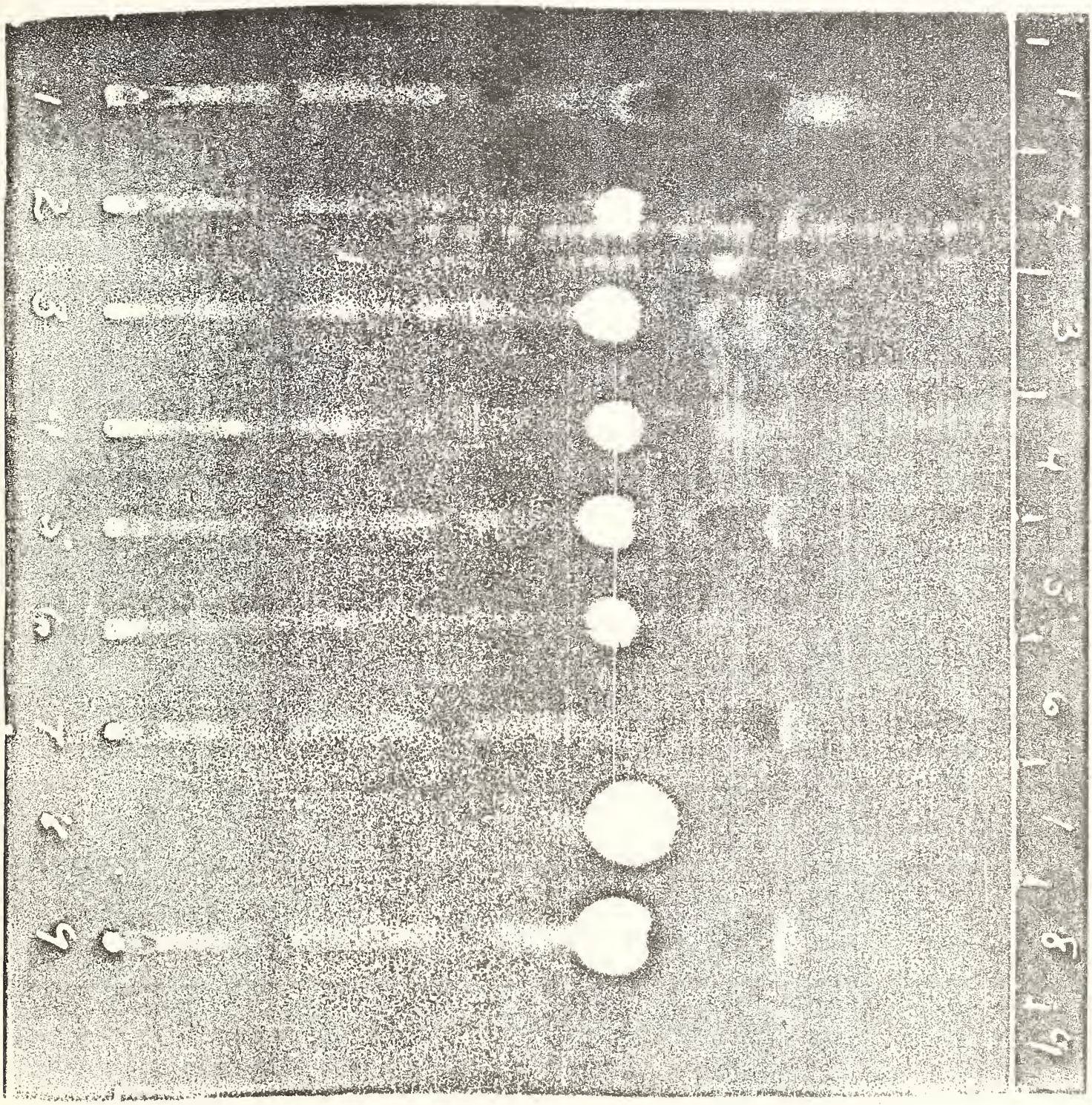
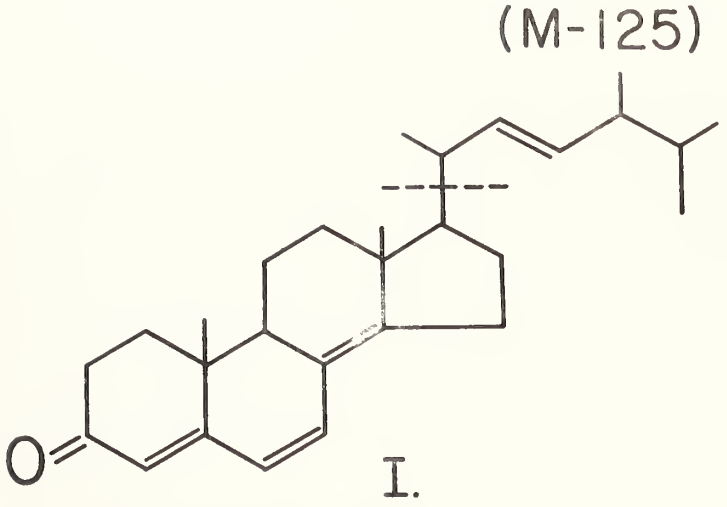
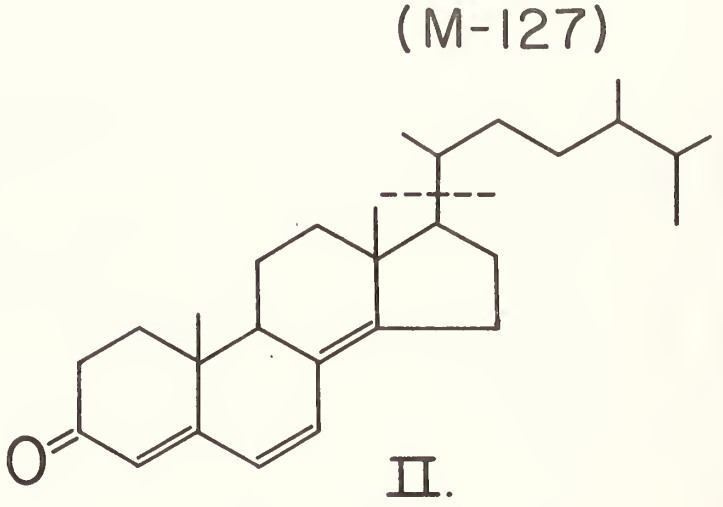


Figure 10. Structures of the ergostaenes, the indicator steroids.



I.

M=392



II.

M=394

I. Ergosta-4,6,8 (14), 22-tetraen-3-one

II. Ergosta- 4,6,8 (14)-trien-3-one

Figure 11. Ultraviolet absorption spectra of ergostaene I, M = 392 and ergostaene II, M = 394 in ethanol (95%).

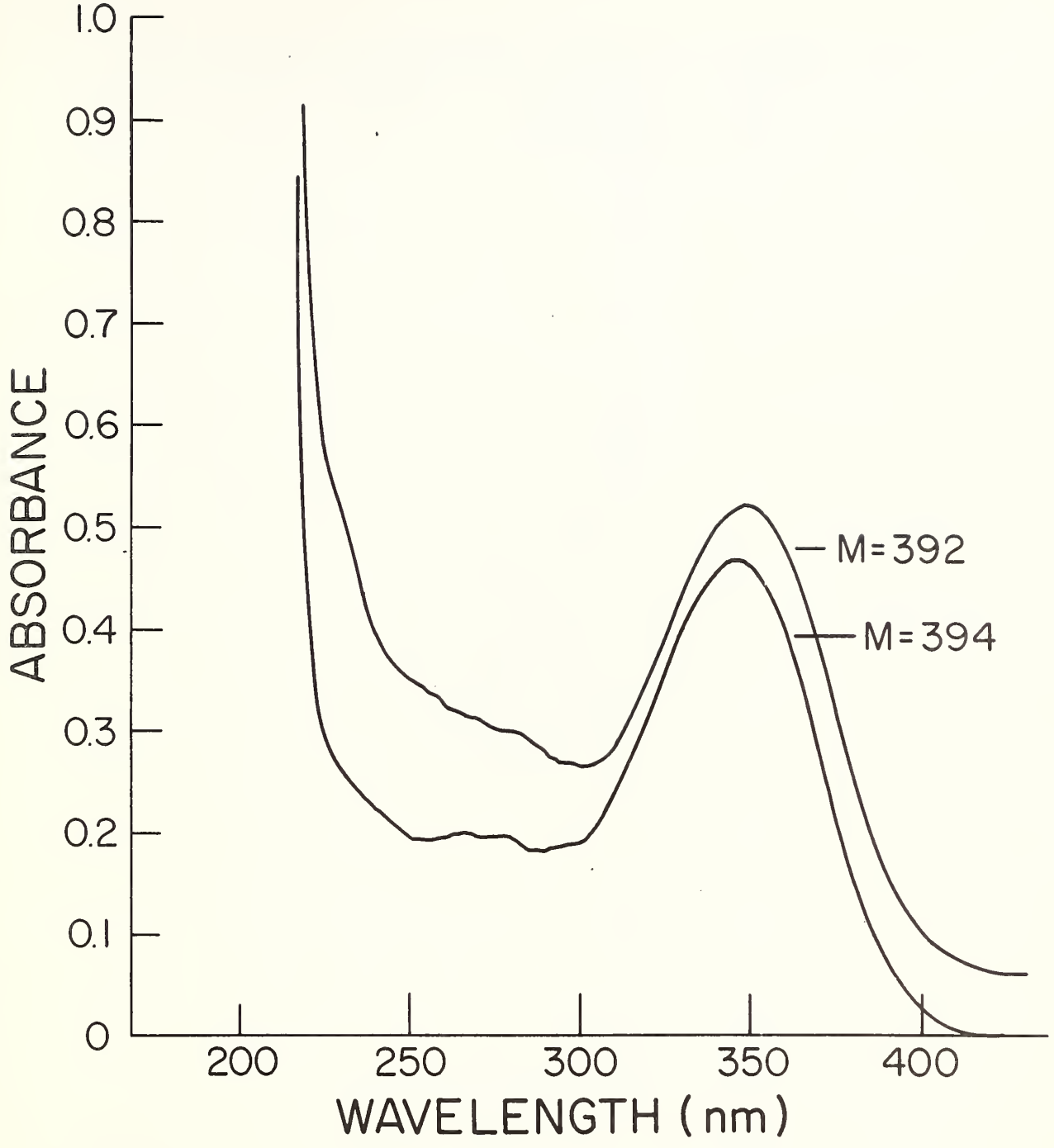
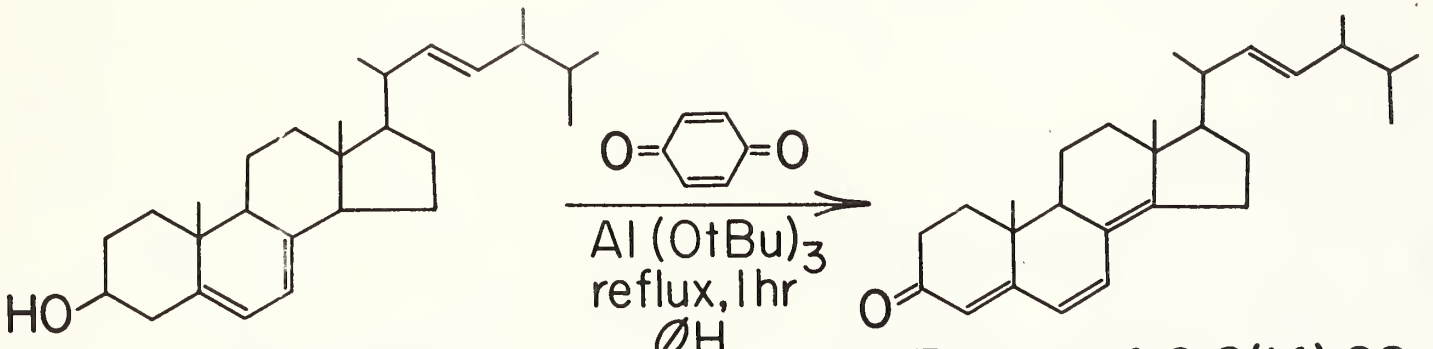

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
200
250
300
350
400
WAVELENGTH (nm)
M=392
M=394

Figure 12. The synthesis of ergostaene I from ergosterol according to Elks, J. Chem. Soc. 1954, 468.



Ergosterol

Ergosta-4,6,8(14),22-tetraen-3-one

J. Elks - J. Chem. Soc. 1954, 468.

Figure 13. Infrared spectra (KBr) ergosta-4, 6, 8(14), 22-tetraen-3-one (I); (A) Blank KBR, trace water OH 3450 cm^{-1}; (B) Natural product from toxic Cynodon dactylon, Louisiana, 1971; (C) Synthetic compound I.

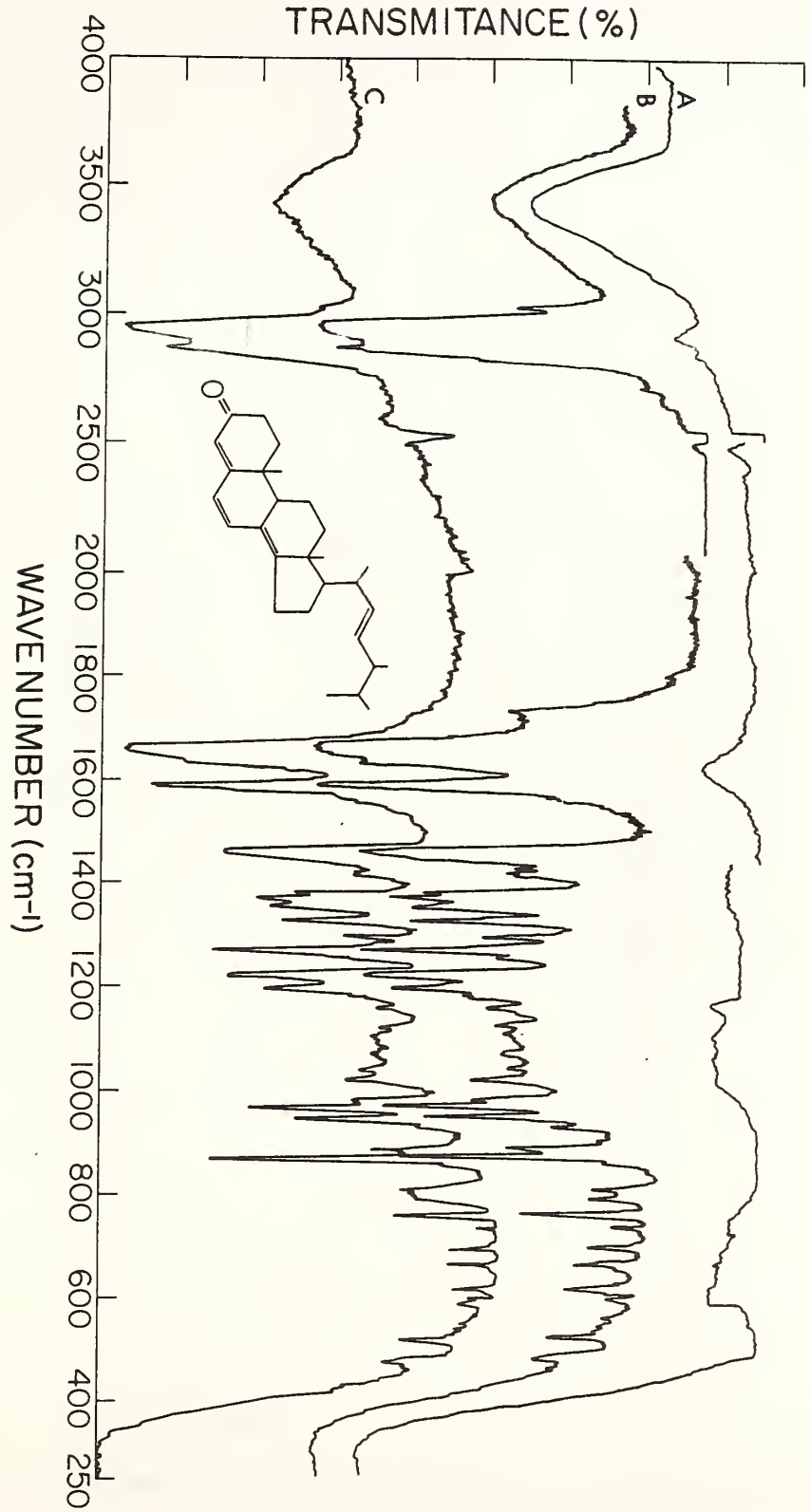
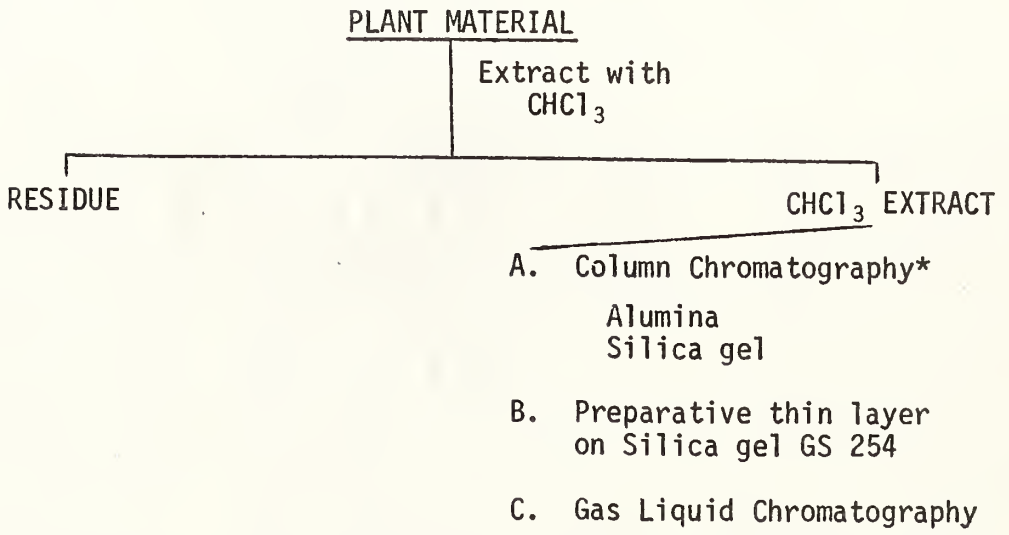

TRANSMITANCE (%)
A
B
C
O
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
250
WAVE NUMBER (cm-1)

Figure 14. Summary of the procedure used to extract the ergostaenes from forages.



*During elution pale blue fluorescent band was followed with UV light at 366 nm.

HIGHLY SENSITIVE METHODS FOR DETERMINING AFLATOXINS

Walter A. Pons, Jr.
Southern Regional Research Center

Highly sensitive methods for determining aflatoxins are valuable in research on the transmission of aflatoxins into meat, eggs, and milk. At the Southern Regional Research Center we developed a method for determining aflatoxin M_1 in milk products such as fluid milk, evaporated milk, nonfat dry milk and cheeses, sensitive to about 0.1 μg/kg (ppb). This procedure utilizes a novel cellulose-aqueous methanol partition column which we find to do an excellent cleanup of extracts prior to thin-layer chromatography. It is also quite useful for cleanup of problem extracts other than milk products. With minor modification we have used this technique to determine aflatoxin B_1 in eggs down to levels as low as 0.03-0.05 ppb. Details of the method for M_1 are attached to this report.

The FDA New Orleans District Mycotoxin Laboratory under the direction of Martin Goldstein has used this method to survey some 320 samples of nonfat dry milk, cottage cheese and evaporated milk from 105 establishments in 18 states. Their concern was due to the known carcinogenicity of aflatoxin M_1 and the widespread utilization of milk in the diets of children. Even though the degree of metabolic conversion of ingested B_1 to M_1 in the milk of cows is in the range of 1-2%, the survey was necessary to determine if a serious problem exists in domestic milk supplies. Briefly summarized, they found detectable M_1 in 15/209 (7%) of cottage cheese, 8/93 (9%) of nonfat dry milks and 1/18 (6%) of evaporated milks. M_1 levels, calculated back to the fluid milk basis from which the products were made, ranged from 0.05-0.4 μg/liter (ppb). The FDA Division of Toxicology concluded that while these levels are not high enough to justify regulatory action, there is reason to want to reduce the incidence of this undesirable metabolite in milk.

Subsequent to publication of the M_1 method, Robert D. Stubblefield, NRRC, who is AOAC Associate Referee for aflatoxin M_1 evaluated the procedure for a wide variety of dairy products such as cheddar, colby, Swiss, cottage, Queso blanco, and ricotta cheeses and butter. He modified the SRRC method for applicability to a wider range of dairy products. He also prepared cheeses from M_1 contaminated milk by established procedures and determined the distribution of M_1 in the curd and whey fractions. His results were published in JAOAC 57 847-851 (1974).

Mr. Stubblefield and Gail M. Shannon of NRRC conducted an international collaborative study of the SRRC-NRRC method for M_1 in milk products involving 19 laboratories in the U.S. and nine other countries. The results were published in JAOAC 57, 852-857 (1974). Based on results from this study the method was adopted as an official first action method of the AOAC for M_1 in milk products 26.079-26.083 (12th Edition, AOAC Methods of Analysis).

Some mention of the precision of M_1 determination should be made. Pons, Cucullu, and Lee (JAOAC 56, 1431-1436 (1973)) obtained a coefficient of variation (relative standard deviation) of 16% for 6 analyses of fluid milk spiked with M_1 at 0.2 µg/L. An experimental naturally contaminated freeze dried milk, with mean of 34 µg/kg, gave a coefficient of variation of 4% (6 analyses). In their international collaborative study, Stubblefield and Shannon (JAOAC 57, 852-857 (1974) obtained coefficients of variation ranging from 45-56% for contaminated samples of milk powder and cheeses ranging from 0.5 to 2.8 µg/kg of M_1 and coefficients of variation ranging from 65-75% for liquid milk and cheese with added levels of M_1 in the range of 0.1-0.5 µg/kg. These M_1 levels are very low and the precision at such levels is good when viewed in the context of the precision of previous AOAC collaborative studies at much higher levels. A calculation of the mean coefficient of variation in five AOAC collaborative studies from 1966-1970 on determining aflatoxins in peanut, copra and cottonseed products showed the following trend. For naturally contaminated products ranging from 10-196 ppb of B_1, coefficients of variation ranged from 10-65%, with mean of 32%. For spiked samples, 10-110 ppb of B_1, coefficients of variation ranged from 17-64%, with mean of 36%.

It is recognized that there is room for improvement in the precision and accuracy of aflatoxin determinations. I personally feel that the inability of different laboratories to consistently reproduce thin-layer chromatographic separation of aflatoxins from nonaflatoxin artifacts in analytical extracts is primarily responsible for the less than desirable precision. All who are in the area of mycotoxin methodology are well aware of the vagaries of thin-layer separations. It is for this reason that SRRC and other laboratories are seriously exploring the use of high pressure liquid chromatography as a more accurate and precise quantitation procedure for mycotoxin determination. Our results so far are promising, and it is anticipated that precise HPLC methods for mycotoxins will be an actuality in the near future.

ASPERGILLUS TOXINS OTHER THAN AFLATOXINS

SLIDE PRESENTATION

by

Richard J. Cole
National Peanut Research Laboratory
Dawson, Georgia

SLIDE No. 1

I want to present to you the -

(1) Objectives of our research program at the NPRL.

(2) The approach we are using to attain these objectives.

(3) Some results of our research program to date.

SLIDE No. 2 OBJECTIVES - OVERVIEW

(1) Determine what molds or other microorganisms are found contaminating peanuts.

(2) Determine which of these microbes are capable of producing toxic chemicals.-

Note: Must be toxic orally to a vertebrate animal.

Isolate and identify the toxic chemical(s).

(3) Note the relative frequency of occurrence of individual toxin-producers from peanuts obtained from various sources over several years.

(4) Determine if toxins are produced on a peanut substrate.

In defense of our approach, I would like to point out that it would be a monumental task to screen peanuts for the presence of mycotoxinsif you have no basis for determining which ones to study.

There are at least 3 - 4 volumes on known toxic metabolites produced by fungi - not counting the toxins that are as yet unknown. Also, these mycotoxins are probably present in minimal amounts; therefore, accurate, sensitive detection methods are required to screen for them as natural contaminants. To just start empirically checking for toxins would be very impractical.

I'd like to now discuss in more detail the various phases of our program.

We first isolate the fungi from peanuts obtained from different sources, during different growing seasons. The fungi are isolated in the normal fashion, i.e. surface sterilization with sodium hypochlorite followed by incubation of an appropriate bacteriological medium. We use Czapek solution agar, Malt-salt agar, or potato dextrose agar. Once these molds are obtained in pure culture, we determine which of these are toxigenic. This is done by growing the fungus on any of a number of media: i.e. shredded wheat supplemented with mycological broth and 15% sugar, corn, rice, peanut meal, or black-eyed peas.

The fungi are cultured on these media for approximately 10-14 days at room temperature. At the end of this time we place chloroform into the flasks, being careful to avoid removal of the cotton plug. The chloroform is heated to reflux before the cotton plug is removed.

The reason we handle the cultures in this manner is that we are dealing mostly with fungi that are capable of producing large numbers of spores. Chloroform precipitates the spores and inactivates the spores, making them safer to handle. The culture is then extracted in a blender and filtered. The chloroform extract is ultimately suspended in an appropriate carrier, i.e. corn oil, and dosed to our experimental bioassay organism. We also fed the residue, which is not soluble in chloroform, to our bioassay organism. In this manner we can safely determine if any toxins are produced that can be extracted with chloroform or if some other extracting solvent is necessary.

The bioassy organism we have selected is the one-day-old DeKalb cockerel. The reason for this selection is availability and cost. We can usually obtain one-day-old cockerels twice a week at a cost of less than 5¢/chick. These animals have demonstrated sensitivity to the various known and many unknown toxins.

SLIDE No. 3

This slide shows the physical facility we use to harbor our chicks during tests. These are incubators that have been modified for our own benefit. We have empirically determined that the extract from 100g of medium cultured in a 500 ml flask is sufficient to dose to 5 cockerels which fit nicely into these compartments.

Let me back up a bit and say that prior to dosing the crude extracts, we routinely chromatograph them on three different TLC systems. This does two things for us. Once we become familiar with known toxins, these can usually be identified and noted at this stage, eliminating the need to dose them to our bioassay organisms. Also, when fungi are isolated from a particular lot of a commodity, many duplicate strains are isolated that cannot be identified as such by just gross appearance of the cultures. We can eliminate dosing of these duplicate strains by determining which of the fungi isolated are duplicate strains.

SLIDE No. 4

This slide presents the results of a typical screening test showing the type of data obtained. The category we are interested in is the last one which shows 5/5 animals showing the clinical signs, whether they be deaths or tremors or what have you.

SLIDE No. 5

This slide shows the clinical signs mostly encountered. Once a particular mold has been shown to be toxigenic at the level required - the test is duplicated to make sure the toxicity can be demonstrated again before we attempt to mass produce the toxin. If the response is repeatable, we place this into a category of a potentially new toxin. We have, in the past, had fungi producing known toxins, ones we were not familiar with at the time or did not have standards for, but this can be determined at a later stage.

The mold is then grown on a large scale, perhaps 100 Fernbach flasks containing 400g of medium. The toxin is extracted with an appropriate solvent and, armed with any of a number chromatographic techniques, i.e. silica gel, Sephadex, ion-exchange, HPLC, Florisil etc., the toxic metabolite(s) are purified using initially the bioassay organism. All through the purification attempts are made to correlate the toxicity with a chemical or chemicals on TLC. Once this is done, subsequent purifications can be monitored with TLC standards.

SLIDE No. 6

This slide is of the Sephadex column.

SLIDE No. 7

This is a slide of above attached to automatic fraction collector.

SLIDE No. 8

This is a slide of HPLC.

SLIDE No. 9

If we do everything right, we may end up with a product that looks like this - Slide of crystals.

Once we have the chemical purified, we obtain the various analytical data, UV, IR, Proton NMR, ^{13}C-NMR, Low-High Resolution Mass Spectra, melting point, etc. A comparison of these data with those in the literature provides us with one of three possibilities:

1st A known toxin, now also known to us.
2nd A known chemical not known to be toxic.
3rd A new toxic metabolite.

If we have a new mycotoxin that is going to be difficult to determine its chemical structure using the analytical data already collected, and if it forms adequate crystals, we rely on single crystal X-ray diffraction analysis to determine the structure.

At this point, I would like to present some of the results of our mycotoxin program.

SLIDE No. 10

This slide shows the chemical structure of a tremorgenic metabolite we isolated initially from P. verruculosum, and it has subsequently been shown to be produced by A. caespitosus and A. fumigatus.

This tremorgen is composed of 6-O-methyl tryptophane, proline, and two isoprene moities. It has a novel 8-membered peroxide ring system that makes it relatively unstable to light.

SLIDE No. 11

This slide shows a related tremorgen called fumitremorgen A. FA was originally reported by Yamazaki, et al. From A. fumigatus, we have recently determined the chemical structure of FA as shown here. It is verruculogen with an addition isoprene moiety at position 13.

SLIDES No. 12 and No. 13

These two slides show related chemicals TR-2 and fumitremorgen B. The structure of FB has recently been reported by Yamazaki, et al.

SLIDE No. 14

This slide shows the structure of tremorgenic metabolite we isolated from P. paxilli. It again contains the basic indole moiety, four additional rings, one of which is a pyrone ring, and an isoprene moiety. This tremorgen crystallized with one molecule of acetone H-bonded to the indole nitrogen. This tremorgen showed low lethality to experimental animals in pure form; however, it still caused sustained tremors in chicks and some additional effects in mice.

SLIDE No. 15

This slide shows a toxic metabolite we isolated from a mold identified as Nodulisporium hinnuleum. It is related to a previously reported metabolite designated viridiol which contains an O-methyl group at this position. Then this compound would be desmethoxy viridiol. This toxin had an oral LD_{50} of 4.2 milligrams/kg in cockerels. It also produced plant-growth regulating and phytotoxic effects. This toxin has a basic steriodal type structure, a phenanthrenene nucleus. It has two ketone functions, two OH functions, and quite an extended conjugation system.

This next metabolite was identified as a previously reported metabolite ID. (1944) but not known to be toxic. This is oospoprein from Chaetomium trilaterale.

SLIDE No. 16

This slide shows the structure of a rather novel fungal metabolite designated moniliformin orginally isolated from Fusarium moniliforme and subsequently by Dr. E. C. White in New Zealand from F. graminarium. This metabolite occurs naturally as either the sodium or potassium salt. The LD_{50} of moniliformin dosed orally to cockerels was 4.0 mg/kg, and again this metabolite also produced phytotoxic and plant growth-regulating effects.

SLIDE No. 17

This slide shows one of the many known toxins we have encountered - decumbin or brefeldin A. I included this metabolite for two reasons. We have encountered it from three different fungi, three different genera. One of the fungi was identified as Neocosmospora africana and apparently this fungi has never been isolated in North America and only three times in the world. I also included it because of the unusual effects it had on our cockerels. The chicks took on this peculiar position with the head held back and, in addition, they moved their heads from side to side in a manner reminiscent of a mating dance.

SLIDE No. 18

This slide shows some of the toxic isolates and respective toxins we have identified. Note the highest frequency was A. flavus and the aflatoxins. The next highest frequency was Penicillum sp. producing citrinin. I might mention that the A. flavus - toxin flavutoxin, the one we are currently studying, may be an important mycotoxin in peanuts.

MYCOTOXIN RESEARCH AT VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

by

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Mycotoxin research at Virginia Polytechnic Institute and State University may, like Gaul, be divided into three parts. Dr. Campbell and his research group are investigating the metabolism of aflatoxin in various systems, while Dr. Griffin is concerned with factors affecting the growth of Aspergillus flavus in the field. Drs. Kingston and Vercellotti are studying the metabolites of Aspergillus versicolor. We will discuss each of these areas in turn.

The metabolism of aflatoxin

A study of the metabolic fate of aflatoxin B_1 in lactating dairy cattle showed that the excretion of aflatoxin M_1 increases to day 4 of the treatment period, and rapidly decreases after cessation of treatment. A large dose of labeled aflatoxin B_1 (1250 ppb) was found to be distributed in the kidney and liver (as B_1 and M_1), in the spleen and bile (as B_1) and in the mammary gland (as M_1). Most radioactivity, however, was found to be present as chloroform-insoluble conjugates.

An in vitro study of the metabolism of aflatoxin B_1 in the bovine liver indicated that it was converted to aflatoxins Q_1 (8%) and M_1 (8%), together with a spot at the origin of the TLC plate (50%) and non-fluorescent materials (28%). No aflatoxins P_1 or B_{2a} were detected.

The microsomal degradation of aflatoxin B_1 has been studied using rat liver microsomes. In the presence of NADPH, aflatoxin B_1 is bound as a metabolite with an absorption peak at 398 nm. Reversible binding to give a complex with an absorption maximum at 360 nm also occurs.

The degradation of aflatoxin B_1 in human liver (two samples, one biopsy and one autopsy) has also been studied. Although differences were observed between the samples, the major chloroform-soluble product was aflatoxin B_1 (12-15%) with aflatoxin M_1 (0.6-1.1%), P_1 (0-1.3%) and two unidentified metabolites also being detected.

Aflatoxin excretion in the urine of human subjects known to have ingested aflatoxins was studied. A small amount of aflatoxin M_1 was detected, but the majority of the B_1 ingested appears to be excreated as non-toxic metabolites or conjugates.

Finally, extensive studies have been made on the role of nutrition as a factor in the metabolism of foreign compounds.

Conditions affecting the growth of Aspergillus flavus

Field work has been carried out on the population of A. flavus in Virginia peanut soils. It has been found that certain cultural practices (e.g. the winter planting of rye) may lead to increased growth of A. flavus.

The nutritional requirements for spore germination of A. flavus have been studied under gnotobiotic conditions and in soil. It appears that mechanical injury of the peanut fruit leads to increased exudation of glucose and amino nitrogen, which leads to increased germination of A. flavus, leading in turn to increased colonization.

In addition to the above work on A. flavus, the peanut black rot fungus (Cylindrocladium crotalariae) is also under investigation.

Metabolites of Aspergillus versicolor

The common fungus A. versicolor is known to produce the anthraquinone pigments versicolorin A, B, and C, averufin, avermutin, and versiconol, as well as the xanthone sterigmatocystin and several related compounds. Because of the similarity in structure between aflatoxin, versicolorin A, and sterigmatocystin, and because averufin and versicolorin A have been implicated in the biosynthesis of aflatoxin, it is necessary to obtain detailed toxicity data on these compounds. In addition, it is of interest to isolate and identify any other toxic metabolites of the mold.

Studies to date have yielded information on the best conditions for growing A. versicolor on rice, together with a protocol for extracting the rice and isolating several of the desired compounds in reasonably pure form. An analytical method for the pigments has been developed using high pressure liquid chromatography: a solvent system of hexane:n-propanol 197:3 has been found to be effective when a small particle silica gel is used as the absorbent. In addition to the named compounds, the new compound dimethylnidurufin has been isolated, together with demethylsterigmatocystin, The isolation of further compounds is currently in progress.

Šession II

A N I M A L F O O D S

INACTIVATION OF AFLATOXIN

by

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INTRODUCTION

Aflatoxin is nearly 15 years old. In 1961 the toxic substance responsible for Turkey X disease in England in 1960 was identified as a metabolite of Aspergillus flavus and was named aflatoxin. However, aflatoxin has undoubtedly been an unrecognized contaminant of molded feed or food products for a much longer time. Raper and Fennel (1965) introduced the preface to their book "The Genus Aspergillus" with the following statement: "The genus Aspergillus dates from Micheli's "Nova plantarum genera" of 1729, but it was not until the 19th century that these fungi began to be recognized as active agents in decay processes, as causes of human and animal diseases, and as fermenting agents capable of producing valuable metabolic products." The species flavus was recognized as early as 1809.

In the past 15 years there has been a tremendous volume of research on aflatoxin. Bibliographies by Boudergues (1969-72) listed 2,191 references to publications through 1972. I have estimated that there must now be over 3,000 publications dealing with aflatoxin in the scientific or technical literature. There is a great deal known about aflatoxin, but thus far we do not know how to prevent its development in the field in agricultural crops such as cottonseed, peanuts, and corn. Fortunately, its occurrence or intensity in most years has been limited to certain production areas. Where unusual outbreaks of aflatoxin occur, this seems to be related to adverse climatic conditions during the growing season or to heavy insect infestation, both difficult for the grower to control.

Figure 1 shows twelve of the fifteen or so aflatoxins which have been identified; these are metabolites of the molds or metabolic products of their ingestion by animals. Contaminated cottonseed usually contains only aflatoxins B_1 and B_2, whereas contaminated peanuts usually contain B_1, B_2, G_1, and G_2.

At the conference of collaborators from the Southern Agricultural Experiment Stations held in 1971, I presented as a coauthor with Dr. Goldblatt "Progress on Control of Aflatoxins and Elimination from Agricultural Products". (Goldblatt and Dollear 1971).

Today I would like to tell you about some of our more recent research on inactivation of aflatoxin in cottonseed and peanut meals which contain levels above the guideline established by the Food and Drug Administration and to bring you up-to-date on the status of this work.

Chemical Inactivation

The approach which we have examined most extensively is chemical treatment to destroy or inactivate the aflatoxin present in contaminated oilseed meals. Having screened a number of different types of chemicals, (Mann et al. 1970) we found the most promising chemical treatment to be use of anhydrous ammonia under pressure. In our early pilot plant work conditions were investigated and it was found that aflatoxin could be reduced from a high level to levels below the guideline using ammonia pressure of about 45 pounds per square inch in the treatment of meal having 12 to 15% moisture content for about one-half hour at temperatures of the order of 200°F.

This was followed up by ton lot treatments (Gardner et al. 1971) at Ranchers Cotton Oil in Fresno, California, and sufficient meal was prepared for animal feeding tests, including a life-time two-year rat study at the Western Regional Research Center, Albany, California. In the cottonseed meal used in that study, aflatoxin levels of about 600 ppb were reduced to about 5 ppb in the treated meal; in one case a nondetectable level was achieved. The Fischer strain of rat was used since it is a strain reported to be susceptible to aflatoxicosis. The cottonseed meals were fed adlibitum in diets containing 20% levels of the meals. In addition to the ammonia "detoxified" cottonseed meal, controls were the aflatoxin contaminated meal, an aflatoxin free cottonseed meal, and the same meal ammoniated under similar conditions. At the conclusion of the feeding test and pending results of histopathological findings, Dr. A. N. Booth reported to us that it was tentatively concluded that the ammonia "detoxified" cottonseed meal did not produce any evidence of aflatoxicosis such as was observed in both sexes of rats fed the aflatoxin contaminated cottonseed meal. I would like to quote him. "In other words, the ammoniation process appears to be a successful means of detoxification." I am pleased to report that we have recently obtained completed data on histopathology of rat tissues from Dr. M. R. Gumbmann, Acting Research

Leader, Pharmacology Research Unit of the Western Regional Research Center. Dr. Gumdmann succeeded Dr. Booth in this capacity upon Dr. Booth's retirement. To quote Dr. Gumbmann: "The only lesions associated with the treatments were liver neoplasms in the positive control group fed unammoniated aflatoxin contaminated meal. Other lesions were termed "spontaneous" in that there was no association to any particular diet. Thus as concluded earlier, ammoniation is shown to be effective in detoxifying meal containing aflatoxin."

Payne, et al. (1972) and Reid (1972) have reported on results of feeding ammoniated cottonseed meal to chickens. At levels used in practical rations laying hens produced well.

You are probably aware that aflatoxin contamination of feed has been of concern with regard to animal safety and economic production of meat, dairy, and poultry products, but it is also of concern to the Food and Drug Administration that animal products from animals fed aflatoxin contaminated feeds might contain aflatoxin or its metabolites which could be a hazard to human health. The most frequent occurrence of aflatoxin in cottonseed is in a geographic area where markets for cottonseed meal are primarily in feed for laying hens producing market eggs. The Food and Drug Administration desired further proof that ammonia "detoxified" cottonseed meal would not be a hazard to human health and safety owing to consumption of meat or eggs from laying hens fed such products. In cooperation with the National Cottonseed Products Association, the U.S. Department of Agriculture developed in consultation with Food and Drug Administration officials a protocol for testing ammonia "detoxified" cottonseed meal. This resulted in a contract jointly supported by the NCPA and the USDA for long-range feeding tests to meet requirements of the Food and Drug Administration. The contract was negotiated with Research 900, Ralston Purina, St. Louis. It involves essentially three phases: First is the feeding of laying hens for a ten-month period to establish animal safety. These laying hens will be observed for egg quality and productivity as well as health. Eggs from hens will be tested for hatchability which FDA officials felt would be a sophisticated test for freedom from aflatoxin in the egg, since any contamination of the egg with aflatoxin would be expected to adversely affect hatchability. In the second phase, laying hens would be fed experimental diets for 60 days, and meat and eggs from these hens would be processed and fed to rats over a lifetime period. Observations of these rats will be made including histopathology of tissues. In the third phase a two generation rat reproduction study is required in which reproduction will be established and the fetuses will be examined for teratology. The dietary plan is similar to that for the earlier rat feeding study, that is incorporating in the diet an aflatoxin-free cottonseed meal, the same meal ammoniated, an aflatoxin contaminated cottonseed meal, and the same meal "detoxified" by ammoniation.

It was agreed that providing meals processed under defined conditions would be the responsibility of the National Cottonseed Products Association. As it developed, this required considerably more effort than had been anticipated. First of all, it was necessary to locate cottonseed that when processed would provide a meal containing of the order of 300 ppb of aflatoxin. An intensive survey of the industry was conducted in the areas where aflatoxin contamination might be suspected and after much effort they were able to locate suitable seed for this purpose. The processing of this meal did not go as smoothly as expected owing to the fact that the plant at which it was to be carried out was a new plant and startup difficulties developed. It was not until the third trial that success was achieved. We were fortunate to have enthusiastic cooperation of the NCPA and Casa Grande Cotton Oil Mill in Arizona and Chickasha Cotton Oil Co. management allowing us to participate in this meal production and observe conditions during processing. Stanley Koltun and Eric Rayner of this laboratory were one or both present when these runs were being conducted. [The presentation was illustrated by some pictures of the plant.]

In the continuous ammoniation plant cottonseed meal is fed into an upper section which is a puff chamber. This vessel acts as a vapor disengaging space for the meal and for the preconditioner feed lock located on the solids discharge of the puff chamber. The cottonseed meal is fed to the preconditioner through this feed lock. The preconditioner is a vessel about 28 feet long and 6 feet in diameter. Steam is introduced at a pressure of about 12 to 15 pounds in order to raise the moisture content of the meal to 15%. An internal agitator lifts the meal and showers it through the steam in the vapor space. The preconditioner discharges through another feed lock to the ammoniator which operates at pressures up to 50 pounds per square inch. The pressure is maintained with anhydrous ammonia vapor which is dispersed through a series of sparge nozzles. The cottonseed meal is lifted and showered through the ammonia atmosphere. The ammoniation vessel is about thirty-four feet long and seven feet in diameter. Both vessels are equipped for steam heating to raise the temperature to the desired level. The ammoniated meal is discharged through another feed lock into the flash conveyor where ammonia and water vapor are released into the vapor dome of the conveyor. The flash conveyor discharges through a weighted seal door into the meal dryer feed conveyor.

This plant was designed and installed by Blaw Knox, and I understand it is a modification of a vapor desolventizer deodorizer used in processing soybean flour for food use. This division of Blaw Knox has now been purchased by Dravo Corporation.

The discharge from the preconditioner and the ammoniation vessel are controlled by discharge gates at the end of the reaction vessels. The setting of these gates controls the time which the meal remains in the preconditioner or ammoniator vessels.

The aim in this processing was to approach as nearly as possible the conditions which we previously achieved in batch scale operation and which provided cottonseed meal containing no detectable aflatoxin. These conditions were not completely achieved and, surprisingly, it was a cold day in Casa Grande when the successful run was made, and the ammonia pressure was only 37 pounds per square inch. Aflatoxin values obtained on ammoniated product were well below the guideline and averaged around 12 or 13 ppb. I am pleased to report at this time that the feeding tests at Ralston Purina are underway and we are anticipating a favorable response in the test animals.

You mak ask what are the remaining problems with the ammoniation process. First of all under conditions for maximum aflatoxin destruction there is also some decrease in nutritional quality of the meal relating primarily, we feel, to some destruction of cystine. Therefore, further work is needed to determine the conditions optimum for aflatoxin destruction with maximum retention of nutritive quality. The cystine destruction was reported by a group in France working with the ammoniation process (Prevot 1974). They pretty much confirmed our results. We have also observed cystine destruction. It apparently is not a serious problem in rations for laying hens but for broilers it does result in poorer growth response.

In order to carry out further work we plan to use a batch type pilot plant ammoniation vessel which was designed by our engineering group. The ammoniator is a pressure-vacuum rotary reactor which is completely jacketed. Steam is circulated through the jacket to heat the material being treated to desired temperature. The double cone shape assures direct contact between the material and the heated surface so that heat is transferred rapidly by conduction.

The reactor is constructed of stainless steel and has an overall capacity slightly in excess of seven cubic feet and a working or operating capacity of five cubic feet. It is fully jacketed and capable of withstanding 100 pounds steam working pressure. The shell or reactor body is also capable of withstanding 100 pounds internal pressure of full internal vacuum. The charge opening is a 10-inch diameter door equipped with eight swing bolts. A neoprene O ring provides a seal for the door. In the center of the reactor is a 3/8 inch vapor inlet or purge line. Just in back of the

inlet is a similar line for venting and below is a quarter inch thermowell. The speed of rotation is controlled by a variable speed drive capable of varying the rotational speed from 2 to 16 rpm. Stanley Koltun reported at the American Oil Chemists' Society Meeting in Mexico City (Koltun et al. 1974) some results using this reactor in studying conditions for ammoniation of aflatoxin contaminated peanut meal. As an example, I would like to show you some of this data. The peanut meal used in these experiments had a total aflatoxin content of 546 ppb. Fifty pounds of meal were used in each run. The meal was charged to the reactor after having been adjusted to 15% moisture content by adding water to the meal, blending in a Sprout-Waldron ribbon blender, and allowing the meal to equilibrate for 24 hours. The reactor was rotated, steam added to the jacket, and ammonia added to the meal. Runs were carried out at pressure from 10 to 60 pounds. Temperature from 124 to 212°F for times from 10 to 80 minutes. In Table I are shown the effect of different reaction times on aflatoxin content at ammonia pressures of 20, 30 and 45 pounds per square inch. In the first group at 20 lbs pressure time varies from 20 to 70 minutes and aflatoxin content of the ammoniated meal from 29 to 3. The second group shows effect of 20 pounds ammonia pressure at higher temperatures of 190°F and times of 10 or 70 minutes. Increasing ammonia pressure to 30 or 45 pounds does not completely remove the aflatoxin when temperatures are as low as 120. In Table II the effect of different reaction temperatures on aflatoxin inactivation at pressures of 45, 30, and 20 pounds is shown. For peanut meal the value of 30 pounds per square inch pressure, 30 minutes reaction time, and 140°F is considered to be about optimum for minimum heat damage to essentially completely inactivate aflatoxin. We are planning to conduct similar experiments with aflatoxin contaminated cottonseed meal. However, in order to better study the effect of the different variables of time, temperature, moisture content, and ammonia pressure on the reaction we have modified the equipment so that samples may be taken at intervals during a reaction under a given set of concitions.

The nature of the products formed in chemical inactivation of aflatoxins in oilseed meals is basic to understanding the inactivation process, and to support biological feeding studies. Since the SRRC ammoniation process for inactivating aflatoxins in peanut and cottonseed meals shows much promise, work was begun to determine the products formed in chemical inactivation with ammoniation of pure aflatoxin B_1 in a model system.

Aflatoxin B_1 was treated with ammonium hydroxide in a sealed bomb under elevated temperature and pressure, selecting time and temperature conditions simulating those used for treating oilseed

Table I. Effect of different reaction times on inactivation of aflatoxin in peanut meal by ammoniation

NH_3 PRESSURE, psig	TEMPERATURE, °F	TIME, min	AFLATOXIN, ppb
20	144	20	29
20	144	45	12
20	140	70	3
20	192	10	61
20	190	70	ND[1/]
30	125	30	15
30	124	50	11
45	128	10	10
45	126	15	16

1/ None detected

Table II. Effect of different reaction temperatures on inactivation of aflatoxin in peanut meal by ammoniation

NH_3 PRESSURE, psig	TIME, min	TEMPERATURE, °F	AFLATOXIN, ppb
45	20	136	5
45	20	143	2
45	20	191	2
30	50	124	11
30	50	175	ND[1/]
30	30	125	15
30	30	148	ND
30	30	212	ND
20	70	140	3
20	70	190	ND
20	20	126	36
20	20	144	29

1/ ND - None detected

meals. From the crude reaction product four distinct products were detected using TLC and visualization sprays. A major product which comprised about 30% of the crude reaction product was isolated in crystalline form and characterized (Lee et al. 1974). This compound having a molecular weight of 286 has been given the trivial name of aflatoxin D_1. Mr. W. A. Pons, Jr. and his associates have also identified a second product of lower molecular weight, 206 (Cucullu et al. 1974). It is similar to aflatoxin D_1 but lacks the cyclopentenone ring.

The structures of these ammoniation products, and a suggested mechanism for their formation are shown in Figure 2. It is postulated that the product of M.W. 286 arises from opening of the lactone ring of B_1 during ammoniation, formation of an ammonium salt, loss of NH_3, and decarboxylation of the resultant β-keto hydroxy acid to form the M.W. 286 product. β-keto acids, such as acetoacetic acid, are known to readily decarboxylate. Since the product arises from decarboxylation, trivial name aflatoxin D_1 was proposed.

One striking feature of the D_1 structure is the loss of the lactone carbonyl grouping characteristic of B_1, which precludes any re-lactonization, to reform B_1, when such a derivative would be ingested by animals. Using high pressure liquid chromatography (HPLC) they have an indication that there are several other products found when ammonia reacts with aflatoxin. These investigations are continuing.

Another chemical inactivation process which we are researching currently is treatment with formaldehyde in the presence of lime. This could be of interest in the preparation of feeds suitable for ruminants since formaldehyde treatment of proteins delays the release of amino acids from the protein and reportedly makes them more available to the animal. In the case of sheep better growth and better wool production have been reported from formaldehyde treated protein sources. Formaldehyde and lime are very effective in reducing aflatoxin content to levels below the guideline, but generally have not under our conditions produced nondetectable levels of aflatoxin.

As an alternative to chemical reaction, we have investigated extraction of aflatoxin with aqueous polar solvents. The solvent of choice here is an azeotrope of about 87½% isopropyl alcohol and 12½% of water. Preliminary work carried out at the pilot plant of Crown Iron Works at Minneapolis convinced us that this was an interesting approach when carried out as a countercurrent extraction operation and we have now installed in the pilot plant a Crown Iron Works

extractor. Only one run has been made with aflatoxin contaminated cottonseed meal. This run was very encouraging as the aflatoxin level was reduced to less than 2 ppb when extraction was carried out essentially at the boiling point of the azeotrope solvent. Further work to determine optimum processing conditions will be conducted. The extract contains the aflatoxin which was removed. We are looking at chemical inactivation of the aflatoxin in this extract such as treatment with lime or sodium hydroxide. The treated extract might then be used as an additive to ruminant feed for economic disposal. Feeding tests will be needed on this material to determine its value and freedom from toxicity.

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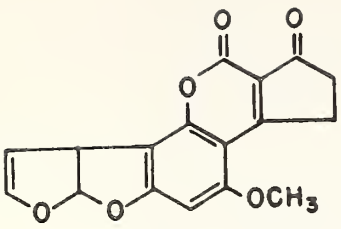
Legends for Figures

Figure 1. Structures of the aflatoxins.

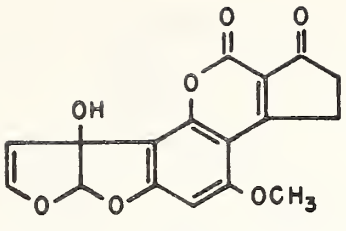
Figure 2. Reaction of aflatoxin B_1 with ammonia.

Photograph

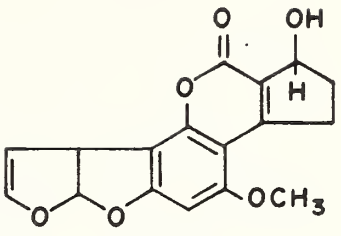
Plant for continuous ammoniation of cottonseed meal at Casa Grande Cotton Oil Mill, Casa Grande, Arizona.



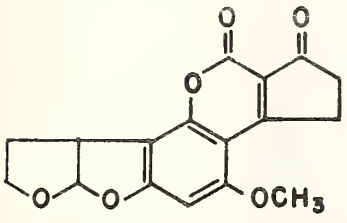
B_1



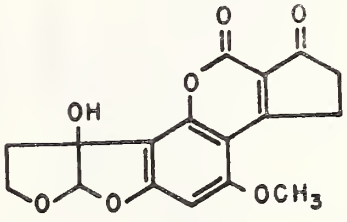
M_1



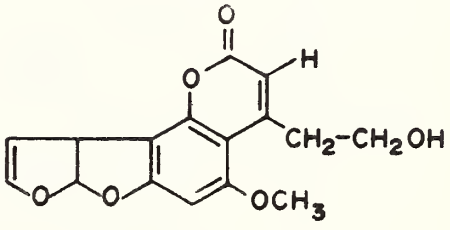
R_0
AFLATOXICOL



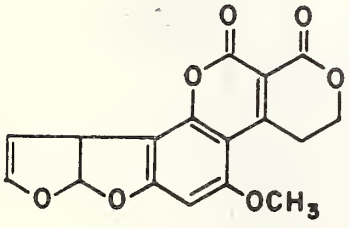
B_2



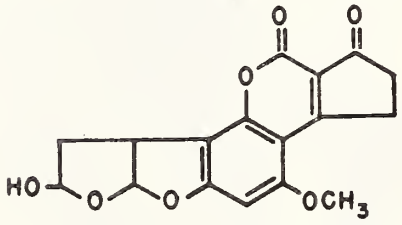
M_2



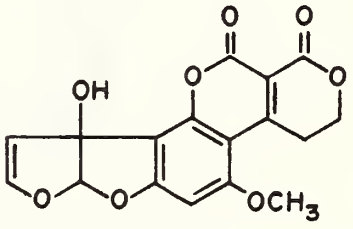
B_3
PARASITICOL



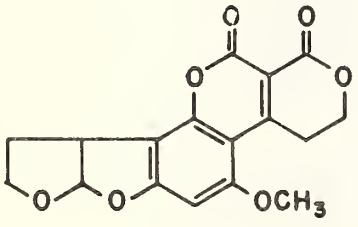
G_1



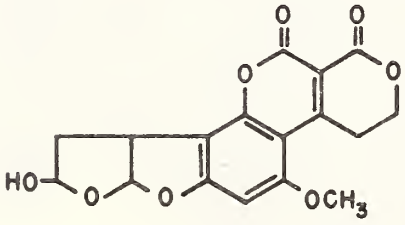
B_{2a}



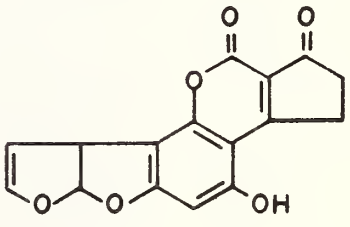
GM_1



G_2

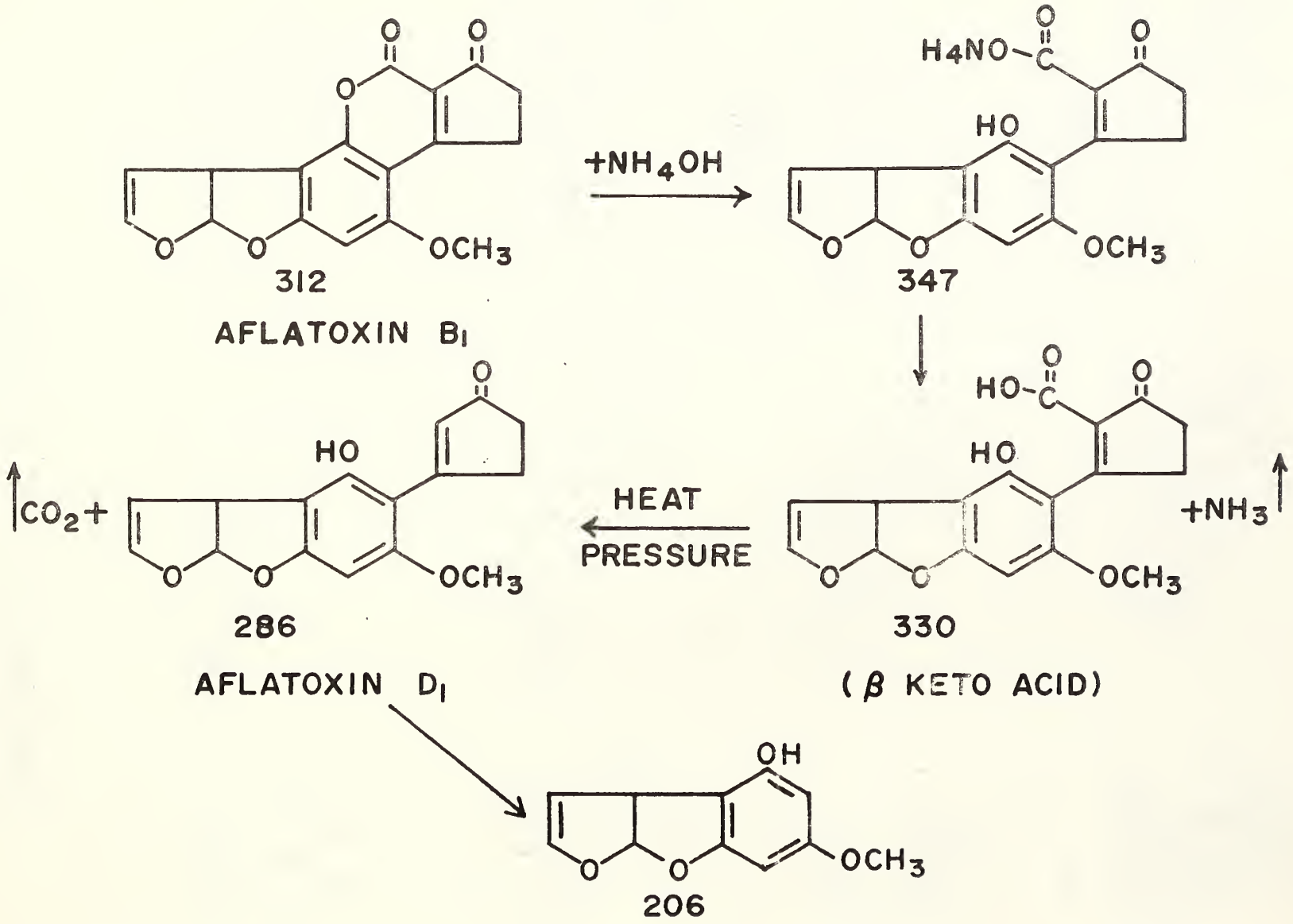


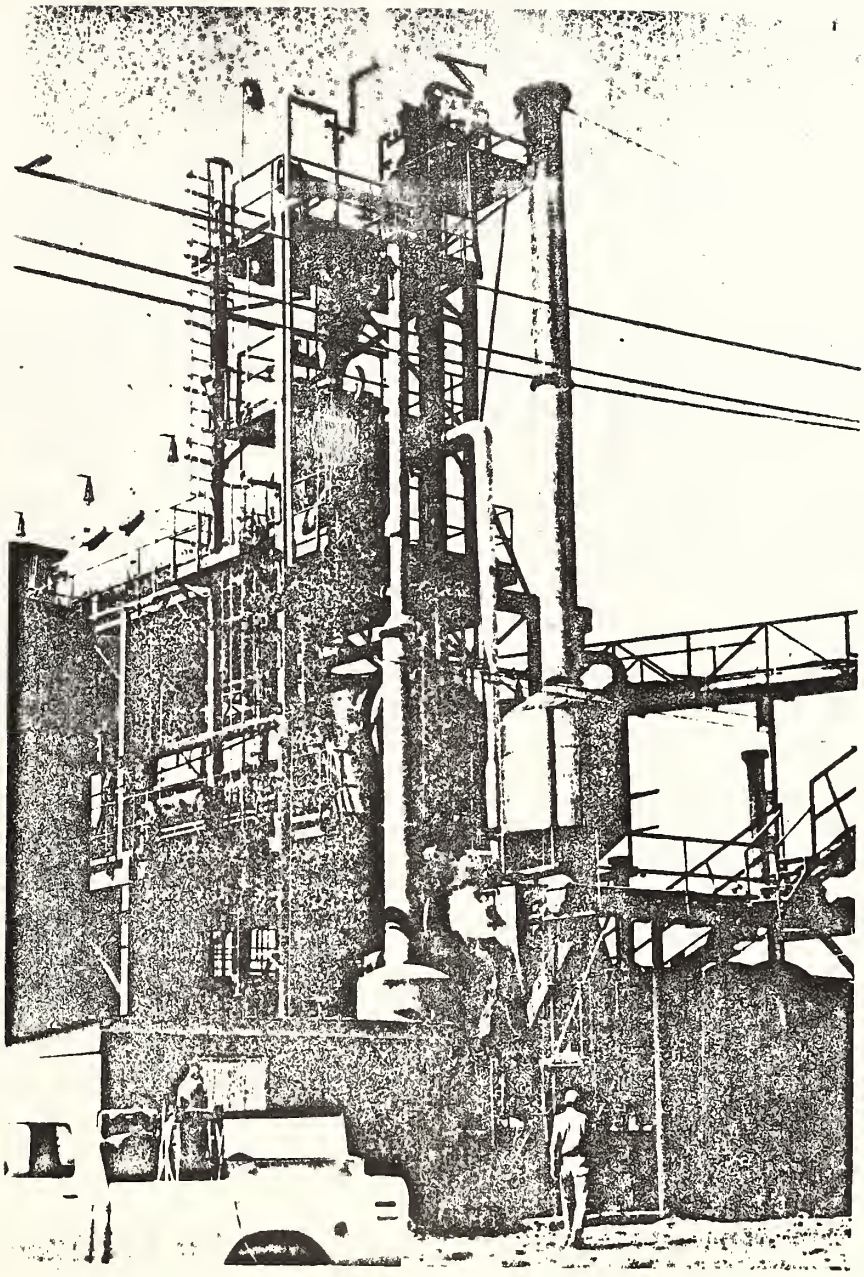
G_{2a}



P_1

STRUCTURES OF THE AFLATOXINS


312
AFLATOXIN B1
+NH4OH
347
HEAT
PRESSURE
CO2+
+NH3
286
AFLATOXIN D1
330
(β KETO ACID)
206



AFLATOXINS IN ANIMALS AND FOOD PRODUCTS

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SUMMARY

Aflatoxicosis caused by aflatoxin B_1 and related toxins represents one of the most serious diseases of poultry, livestock and other lower animals as well as man.

The aflatoxins, produced by approximately 30% of the strains of Aspergillus flavus and by Penicillium puberulum, are frequent contaminants of harvested feeds and foods (rice,corn, sorghum, and other grains; peanuts, cassava, and fermented products) stored under conditions of high humidity and temperature.

Aflatoxins cause severe hepatotoxicosis in many species of animals and carcinogenesis in others. Aflatoxin B_1 may cause hepatomas at amounts as low as 15 ppb in the diet of trout and is one of the most potent carcinogens recognized.

Aflatoxins that accumulate in milk or other tissues from food-producing animals may pose serious hazards to young animals or infants consuming such products.

Mycotoxicosis, later identified as aflatoxicosis, was recognized in the early 1950's when swine and cattle in Alabama, Florida, and Georgia died after eating moldy corn. Lesions were experimentally produced in pigs and calves fed moldy corn obtained from farms where the losses had occurred. Extensive losses in England in 1960 in turkeys and ducklings consuming moldy toxin-contaminated peanut meal were attributed to aflatoxins associated with the presence of Aspergillus flavus, before its complex nature was determined. The aflatoxins B_1 and B_2 produced blue fluorescence on thin layer chromatography plates and G_1 and G_2 produced greenish fluorescence. Since other fractions have been isolated and characterized; the major constituents have been identified as difuranocoumarin derivatives.

The principal toxic action is due to aflatoxin B_1 and affects the liver of susceptible species resulting in icterus and stunting, with bile duct proliferation, fatty changes with vacuolation, and marked fibrosis with more advanced toxicosis. Aflatoxin is recognized as a very potent hepatotoxin and carcinogen. Peanuts, pecans, cassava, bread, country cured hams, cheese, cream and butter, rice, cottonseeds, copra meal, corn and sorghum plus other grains susceptible to invasion by toxigenic strains of A. flavus including stored silage may contain aflatoxins. The aflatoxins are produced by approximately 30% of the strains of Aspergillus flavus and Penicillium puberulum isolated. They are hazards to animal and poultry production and potentially to mankind.

Other nations have also recognized the potential of aflatoxin B_1 hazard to animals and mankind. In Canada, the Food and Drug Directorate does not have a legal tolerance but they have an "acceptable" limit. In France, a high tolerance limit of 700 µg/Kg has been fixed for basic components and 50 µg/Kg for finished feed products. In West Germany, the maximum allowable level in mixed feeds is 200 µg/Kg. The United Kingdom has no legal tolerance for either human or animal food but there is a working limit of 50 µg/Kg for animal feeds. It has been proposed that a limit of 150 µg/Kg be set for the Common Market. In Sweden, in terms of animal feeds or peanut meal for animal feeds, they have a voluntary agreement on a maximum of 600 µg/Kg. In South Africa, an aflatoxin content below 3000 µg/Kg may be incorporated into certain animal rations yielding 800-1200 µg/Kg in the finished feeds.

In Poland, a zero tolerance limit for aflatoxin has been established for the following feeds: for calves up to 3 months, piglets, laying hens, breeding hens, broilers and meat turkeys. Levels of 4 to 80 µg/Kg are permitted in poultry feeds; 4 to 99 µg/Kg in cattle and sheep feeds. Peanut meal can contain from 90-5000 µg/Kg but at the higher levels, only 2 percent of peanut meal can be used in feed mixtures.

Representative aflatoxin B_1 levels in Polish feeds (1974).

Cattle and Sheep - 3% sampled (37) contained 300 µg/Kg.

Poultry - 2% sampled (59) contained 30 µg/Kg.

Swine - 4% sampled (121) contained 1000 µg/Kg.

(-) no. feeds sampled and assayed for aflatoxin B_1.

Feed ingredients, principally peanut meal, were contaminated at levels up to 2000 μg/Kg. Of the mixed feed samples analyzed, 7.3% were contaminated with aflatoxin B_1.

Aflatoxin residues in food of animal origin

As early as 1963, it was suggested that aflatoxin residues might be present in milk and other animal products. Aflatoxin B_1 has a LD_{50} in ducklings of 0.5 mg/Kg; B_2, the dihydro derivative LD_{50} is 1.70 mg/Kg/ aflatoxin M_1 is equivalent to B_1 in toxicity. The LD_{50} of G_1 is 0.8 mg/Kg while that for G_2 is 3.4 mg/Kg. Aflatoxin B_1 is the most potent carcinogen known, total doses as low as 400 μg have produced tumors in male rats. The comparative toxicities to sev eral animal species are presented in Table I. Note the increased susceptibility of young, male animals. The technique of Eppley (1966), or modifications thereof, for determinations for aflatoxin B_1 has been used in the skeletal muscle and livers of chickens. A slight modification of this technique was used (1969) to detect levels of B_1 and G_1 in stored meats. A technique for detection of aflatoxin M_1 in liquid milk has been described.

Aflatoxin M appears in cows' milk within 2 days of ingestion of aflatoxin B_1 contaminated feed, the amount present being 0.5-1.0 percent of that ingested. The toxin content apparently increases in cows receiving B_1 contaminated feed as the daily milk production volume decreases. A level of 0.6 to 0.9 mg/day aflatoxin B_1 consumption is likely to result in measurable levels of M_1 in the milk. Aflatoxin M_1 has more recently been observed in milk up to 0.25 μg/L from high-yielding dairy cows on aflatoxin-containing feeds. Molded silage and hay were also implicated.

Shotwell, 1972, reports on the correlation of bright greenish yellow fluorescence with aflatoxin contamination in individual corn kernels. The kernels were separated from 10 naturally contaminated corn samples. All of the 124 fluorescing kernels examined contained aflatoxin B_1 levels ranging from 4 to 270,000 μg/Kg.

Chickens exposed to aflatoxin B_1 responded with deposition of increases of hepatic fat, reduced vitamin A storage, and fluctuation in the DNA-RNA ratio (Carnaghan, Lewis, 1966). There was some decrease in liver weight in ducklings provided feed containing 0.6 ppm B_1 for 1 week, followed by a significant increase in the liver weight for the next 2 weeks. Some of the livers exhibited increased firmness of texture, petechial hemorrhages, and the first signs of fatty infiltration. After 2 weeks, all the livers of the B_1 ducklings were a pale, yellow ocher color. One week after the aflatoxin B_1 diet was stopped the livers were nearer normal in size with pin-head-sized necrotic lesions. After 2 weeks on the B_1 ration, there was inappetence, dullness, and reduced growth rates.

Research reported or underway by Edds, Simpson, Harms, and Wilson on aflatoxicosis in poultry has demonstrated that quail, turkeys, and New Hampshire chicks are susceptible to aflatoxin B_1 at levels of 200 ppb in the feed. Hybrid, Rhode Island Red, and Leghorn chicks are more resistant. Morbidity and mortality in quail were effectively reduced by adding an antibiotic-vitamin supplement* to the ration. Aflatoxicosis in young turkey poults was also reduced with this supplement when used at the listed therapeutic levels.

Exposure of day-old New Hampshire chicks to a standard started ration containing aflatoxin B_1 at 200 ppb for 21 days with challenge of the survivors with cecal coccidiosis at that time, resulted in a significant increase in mortality of the groups not protected with a coccidiostat. Further challenge of these chicks with Marek's virus resulted in significant increase in severity of lesions at necropsy. These data lend credence to the suggestion that aflatoxin B_1, in addition to the liver damage, also lowers the resistance to parasitic and viral diseases.

A trial with exposure of New Hampshire groups of chicks at 200 ppb or 2 ppm for 28 days followed by a return of the exposed chicks to a standard starter feed for 3 weeks resulted in return

* Floxaid - Merck & Co., Rahway, N.J.

to normal appearance and weight gains. However, the 2 exposed groups were more susceptible to cecal coccidiosis than groups not previously exposed to aflatoxin or exposed and receiving a coccidiostat. Similar results were observed in hybrid chicks exposed at 0.2 and 2.0 ppm aflatoxin B_1.

Tamarelle, et al. described the significant reduction in serum cholinesterase associated with liver cirrhosis and neoplasma and severe malnourishment. Its estimation may be of prognostic value in virus hepatitis and liver cirrhosis. In various disorders, this fall in serum cholinesterase level is often comparable to the fall in the prothrombin levels and serum albumen, but is more constant and more sensitive.

Aflatoxins ingested by food-producing animals may result in residues or metabolites in human foods. These aflatoxins can be detected in edible tissues and milk. The Food and Drug Administration announced in 1969 that a level of more than 20 ppb would not be allowed in feeds. However, there is no tolerance allowable for aflatoxins in any foods intended for human consumption. The government does allow approval of peanuts for food processing at 20 ppb or less if adequate precautions are maintained to further reduce these levels. There have been instances reported where the rejects or pickouts have not been handled properly; they may have been put into peanut meal for animal feed use and thus increase the hazard for animal feeds and ultimately human consumption.

A new phenolic derivative of aflatoxin B_1, appearing mainly in conjugated form, was identified by Dalegros and Wogan as the principal urinary metabolite of aflatoxin B_1 in rhesus monkeys. Its identification in human urine might facilitate estimation of aflatoxin exposure in human populations. Aflatoxin P_1 represents at least 60 percent of the urinary aflatoxin derivatives. Of this, about 50 percent is present as glucuronide, 10 percent as sulfate, and 3 percent as unconjugated phenol. Together these represent more than 20 percent of an injected dose of aflatoxin B_1.

Cows placed on feed containing aflatoxin B_1 had M_1 in the urine, feces, and milk.

Aflatoxin Q_1 is a major metabolite of aflatoxin B_1 when the latter is biotransformed by the monkey or rat liver preparations. It has been identified as an isomer of aflatoxin M_1. In monkey liver

microsomal preparations, as much as 52% of aflatoxin B_1 is converted to aflatoxin Q_1, therefore, it is important to determine the chemical properties, toxicity, and biochemical role of this major aflatoxin metabolite.

Aflatoxin incidence in animal feeds

Aflatoxin was found in 86.5% of 52 samples of peanuts and peanut products imported into Denmark from 10 countries. A field study in North Carolina of aflatoxicosis in broiler feeds revealed that the primary source of aflatoxin in the mixed feed was corn. The corn samples analyzed showed an incidence of 30% toxin contamination. Earlier, Wilson et al. assayed seven corn samples associated with toxic hepatitis in swine and cattle in the Southern United States and found five contained aflatoxin in amounts ranging from 50 to 280 ppb Shotwell et al., 1973 reported 21 of 60 corn samples analyzed in 1969-1970 contained aflatoxin at levels greater than 20 ppb, the governmental guideline.

Mycotoxin analysis for all feed components entering a large Florida feedmill for the year 1971 indicated that aflatoxins represented over 50 percent of the toxins present. Although 16.5 percent of 634 samples had detectable levels of aflatoxin, only 26 of the samples, 4 percent, contained levels greater than 20 ppb, the maximal government allowable level. However, 57 or 8.9 percent contained molds, which under improper conditions of storage, high moisture and temperature, may have developed hazardous levels of aflatoxins.

Numerous aflatoxin positive corn samples submitted from Florida farms where aflatoxicosis had been diagnosed on the basis of clinical signs and gross lesions suggested a need for broad sampling of corn samples from Florida farms. The County Extension Directors were contacted and district meetings held to acquaint them with this problem and methods for detection of aflatoxin. The detection of aflatoxin positive kernels with a long-range fluorescent lamp and confirmation with the minicolumn or thin layer chromatographic methods were demonstrated.

The Extension Directors collected 10 pound samples from 77 farms in the summer and fall, 1974; 14 or 18% were positive at levels of more than 100 ppb. This compared favorably to 19 of 118 corn samples or 16% positive at more than 100 ppb submitted during 1973. Farmers experiencing swine losses from aflatoxin contaminated corn were advised on the importance of buying and storing whole kernel corn in cleaned dry bins at a moisture level less than 14%. Their losses have been dramatically reduced. Corn shipped into Florida with dirty, cracked, or weevil infected kernels has been more highly contaminated with aflatoxin.

Moldy corn toxicoses in swine and cattle were characterized by hemorrhaging in many tissues including liver, esophagus, stomach, intestines, skeletal muscle, and subcutis. The intestines, and sometimes the abdominal cavity of pigs were filled with blood. Similar characteristics included frequent icterus; variable amounts of fatty changes in the liver, visible grossly and microscopically, considerable bile duct proliferation; and hemorrhages in several organs, tissues, and cavities immediately preceding death.

Factors influencing aflatoxicosis

In addition to age, sex, species, and breed variations in susceptibility to aflatoxins, nutritional and disease factors influence the degree of response to these toxins. A diet low in lipotropic factors predisposed to greater hepatotoxicity in rats. Chlortetracycline and vitamins A, E, and K, added to toxic diets containing 0.18 ppm aflatoxin resulted in increased growth rates, but clinical signs, mortality, and histopathologic changes associated with aflatoxins were not influenced. Both 4- and 10-week-old pigs, when fed rations containing high amounts of protein (20.6 to 17.0%) vs. low concentrations (14.1 to 11.4%), developed more serious signs and lesions at the lower concentrations. These signs and lesions included stunting, icterus, with hepatic cell necrosis, hemorrhage, and bile duct hyperplasis, along with hydropic and fatty degeneration of hepatocytes.

Clinical signs and gross and microscopic lesions

Swine - Aflatoxin B_1 provided continuously in feed at concentrations of 0.28 to 0.41 ppm to groups of 40-lb. (18.2-kg.) pigs for 107 days caused depression of growth rates and feed conversion efficiency. Hepatic lesions in 12- to 14-week-old pigs fed rations containing 0.615 or 0.810 ppm aflatoxin B_1 included pale yellow discoloration of the liver, with accentuated lobular patterns. One

pig that died after being given feed containing 0.810 ppm aflatoxin B_1 had icterus, severe gastrointestinal hemorrhage, and bloodstained fluid in all body cavities. Pigs from a sow given aflatoxin at a rate of 0.234 mg./kg. of body weight for four days immediately after parturition and compared with pigs from a control sow farrowing at the same time, were clearly stunted by time of weaning and the stunting persisted until slaughter.

Hansen, Harland, and Rubin, 1971, reported the outbreaks of aflatoxicosis on swine farms in Florida. Veins were so fragile that the normal handling of pigs led to massive, subcutaneous hemorrhage. Corn being fed contained 150 ppb aflatoxin B_1. Addition of vitamin K to the ration reduced prothrombin times to normal levels. The condition was prevented by the addition of 2.2 mg vitamin K per Kg. of ration. Espinasse et al., 1973, reported that piglets consuming a suspect creep feed died of hemorrhagic disorder. Clinical signs included lameness, swelling of the joints of the hind legs, crippling hematomas, and increased prothrombin times.

Feeding of an experimental aflatoxin B_1 contaminated ration, 450 ppb to 28, 5.5 to 7.7 Kg. piglets, for 14 days produced significantly lower daily feed intake and daily gains ($P < .01$) than 28 untreated pigs on a normal ration. On removal from the aflatoxin contaminated feed, the pigs regained the normal rage of gain.

Sheep - Loss of appetite and diarrhea occurred in sheep given aflatoxin at a rate of 0.23 mg./Kg. of body weight and at higher dosages. These signs were accompanied by excessive salivation, tachypnea, and pyrexia at dosages of 0.59 mg. or more per kilogram of body weight.

Cattle - In 1963, Allcroft and Lewis reported their findings on the effects of toxic Brazilian peanut meal for calves, cows, and heifers. In calves the first symptomatic effect of continuous ingestion of toxic peanut meal was a reduced growth rate followed by severe straining a few days before death.

Fifty cross-bred beef steers purchased on the open market at age 6-8 months and in a body weight range of 400-500 lbs. were used. Weight gains were significantly lower when the level of aflatoxin in

the ration was 700 ppb or higher. The feed efficiency values decreased as the level of aflatoxin fed increased. The livers from three of eight steers fed 1000 ppb and two of ten steers fed the 700 ppb level of aflatoxin B_1 were grossly abnormal being greyish in color, enlarged, and having a fibrous or rubbery texture. Below 700 ppb no gross liver abnormalities were observed.

When cows ingest contaminated feeds containing sufficient aflatoxin B_1 to yield a dose of 0.6 to 0.9 mg./day, they excrete the metabolite aflatoxin M_1 in their milk. This causes hepatotoxicity similar to that induced by aflatoxin B_1 in young ducks. Silage samples from moldy silage pits contained aflatoxins B_1 and G_1. Also, 5% of milk samples from cows fed moldy silage contained aflatoxin M_1. This has public health significance. Because, aflatoxin M_1 has an affinity for the casein fraction of milk, the concentration is highest in skim milk.

Twenty-four male Holstein calves, two weeks old and averaging 44 Kg. body weight, were assigned at random to eight treatment groups, seven aflatoxin dose levels, and one control group with three calves per group. The calves were raised in individual pens and fed 3 l. of whole milk daily plus hay and a 12% protein grain ration.

Each animal received a single oral dose of aflatoxin in a gelatin capsule at the beginning of the experiment.

Preliminary work indicated that a single does of the aflatoxin crude powder at levels below 0.6 mg./Kg. body weight was not lethal to calves. The data show that all the calves survived for the 42-day experimental period at dose levels up through 1.0 mg./Kg. body weight. The 1.8 mg./Kg. body weight dose was lethal for all three calves. The average survival time for the calves at this dose level was 23 days.

Further data supporting the suppression of disease resistance by aflatoxin B_1 was presented by Osuna, 1974. Twenty-four male Holstein calves, 4 weeks old and averaging 101 pounds were divided into six groups, with three groups infected with 0 or 220 Fasciola hepatica metacercariae and exposed to 3 levels of aflatoxin B_1 5 weeks later. Three similar groups of 4 calves received only aflatoxin B_1 at 0, 0.5, or 1.0 mg./Kg. as did previously infected calves with the fluke larvae.

This resulted in a significant decrease in dry matter intake, body weight, and serum albumin, increased values of prothrombin time, serum alkaline phosphatase, and serum sorbitol dehydrogenase. Significant differences in the number of flukes recovered from the livers were seen in the groups receiving 0.5 and 1.0 mg./Kg. of aflatoxin B_1 (see Table 2).

In all animals necropsied, the liver was the organ most affected with aflatoxin as well as flukes. Periportal fibrosis, monocytic infliltration, fatty infiltration, and bile duct proliferation were the characteristic lesions produced by aflatoxin B_1.

Additive toxic effects were observed in the groups dosed with flukes and aflatoxin B_1 with significant variations of serum and plasma values as well as severity of histopathologic lesions.

Table I - Aflatoxin B_1 Oral LD_{50}, by Species

Species	Dosage (mg./kg. body weight)
Trout (100 Gm.)	0.5 - 1.0 (10 days)
Duck (day-old)	0.4 - 0.6 (5 days)
Rabbit	0.3
Cat	0.3 - 0.6
Pig (6-7 kg.)	0.62
Pup	0.5 - 1.0
Rat	
Day-old	1.0
21-day old	5.5 - 7.2
Guinea pig	1.4
Sheep	2.0
Chicken	
(Rhode Island)	6.3
Monkey	
(macaque)	7.8
Hamster	10.2
Catfish	
(channel)	10.0 - 15.0 (5 days)

Table 2 - Gross Liver Lesions Observed at Necropsy

Group No.	Calf No.	Liver Fibrosis	Nodular Surface	Fluke Ova	Fluke Nos.	Avg. per group
III	9	-	-		-	
IV	13	*	-	***	19	
IV	14	*	*		5	
IV	15	**	-	****	11	
IV	16	*	-	*	17	13
V	17	*	-	-	58	
V	18	*	-	-	46	
V	19	**	-	****	39	
V	20	*	-	-	15	40
VI	21	**	**	-	48	
VI	22	**	**	-	21	
VI	23	***	***	-	95	
VI	24	****	****	-	208	118

OVERVIEW OF AFLATOXICOSIS IN POULTRY

by

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Aflatoxicosis was first reported as Turkey "X" disease in 1960. This new disease which also affected chickens and ducks as well as swine and cattle was found to be associated with the use of Brazilian peanut meal which was infested with Aspergillus flavus. Toxic principle(s) called aflatoxin(s) were isolated and the scientific world was greatly intrigued when aflatoxin was reported to cause cancer in rats. This finding has motivated much of the work on mycotoxins since then. However, it is an interesting reflection on science as a whole that the toxic principle(s) isolated were never tested in turkeys to see if they caused "X" disease. This failure to fulfill Koch's postulates as they apply to mycotoxins is a recurring theme in mycotoxicology and is manifested more broadly in a paucity of studies on the effects of mycotoxins in animals. North Carolina was "fortunate" in having mycotoxin problems annually in its poultry, in having good facilities to study mycotoxicoses in poultry, and in having an Experiment Station administration farsighted enough to support research on the effects of mycotoxins in poultry. Our efforts have dealt primarily with aflatoxicosis since it is the major mycotoxin problem in poultry as far as we know.

Aside from rare catastrophes such as losing half of a flock in 72 hours from a bin of moldy corn, uncomplicated aflatoxicosis is usually characterized in broilers by poor growth rates, poor feed conversion, increased condemnations, and increased mortality during the grow-out period. These losses can be sporadic or persistent and range from nickel and dime losses to bankruptcy. In a study of a typical case of aflatoxicosis the final body weight was off 1/4 lb., feed conversion ratios were increased from 2.1 to 2.3, condemnations were tripled, and mortality was doubled.

Our first efforts were aimed at developing some guidelines which would help in diagnosing aflatoxicosis. A pale, enlarged, friable, fatty liver is the most obvious symptom seen on necropsy. The spleen and pancreas are enlarged while the bursa of Fabricius is regressed. This has been documented in both laboratory and field studies.

Aflatoxin causes an increased susceptibility to bruising in broilers as measured by the minimal energy required to cause a bruise. This is accompanied by increased capillary fragility and decreased lateral shear strength of muscle. Time studies revealed that capillary fragility was increased significantly only 48 hours after aflatoxin was introduced into the diet. This time response was correlated with an increase in lysosomal enzymes. Aflatoxin also caused an increase in clotting time of blood, thus once a blood vessel is ruptured by a blow the rupture will not be staunched as soon as normally. Aflatoxin, then, is one of the few documented underlying causes for bruising which causes an annual loss of $70 million to the poultry industry.

Aflatoxin interferes with the immune system and makes birds more susceptible to infectious diseases. It impairs the reticulo-endothelial system and it acts as an immunosuppressant. It has been documented to cause vaccine failures and to cause increased losses to diverse diseases such as crop mycosis, paratyphoid, and coccidiosis. These interactions can occur with levels of aflatoxin too small to affect the growth rate.

Aflatoxin also interacts with environmental stresses. It can make birds more susceptible to extremes of heat and cold by interfering with energy metabolism. Aflatoxin is also a nephrotoxin with a dramatic effect on kidney size and function and makes birds more sensitive to dietary sodium.

Aflatoxin has many interactions with nutrition. It effectively increases the protein requirement of birds. Increased fat content in isocaloric diets can spare both the mortality and growth inhibition caused by aflatoxin. Dietary antibiotics can partially protect against aflatoxicosis as would be expected of a toxin that interferes with the immune system. Vitamin deficiencies can interact with aflatoxin to the detriment of the birds. Vitamin D, riboflavin, and pantothenol deficiencies make birds susceptible to levels of aflatoxin otherwise too small to cause an effect.

The performance of laying hens is adversely affected by aflatoxin. It causes a dose related enlargement of the liver, fatty infiltration of the liver, and a decrease in egg production. Aflatoxicosis in laying hens matches the description of "fatty liver syndrome" which has been problem of unknown origin. Aflatoxin causes a decrease in serum proteins, lipids, and calcium which are egg constituents. There is a dose related decrease in egg size. The effect on egg production is time dependent in that there is about a two weeks period after introduction or removal of aflatoxin from the diet before egg production decreases or returns to normal. This makes for a very difficult epidemiology.

Surveys have revealed that aflatoxin can originate anywhere in the feed supply chain from the corn field to the feeding troughs. There is a "snow-ball" effect in that the problem becomes worse the closer the feed comes to the birds. This indicates that control efforts at all points in the supply chain will be beneficial and field cases buttress this viewpoint. The prospects for effective control are bright for the long range, but much additional work will be required. It must always be kept in mind that over 120 mycotoxins have been reported but less than half a dozen have been studied in a decent fashion for their effects in animals. We will undoubtedly find that many problems of currently unknown etiology are the results of mycotoxins and their interactions.

Session III

RAW AND PROCESSED AGRICULTURAL PRODUCTS

MOLD GROWTH AND MYCOTOXIN PRODUCTION ON AGED HAMS AND SAUSAGES AND IN PECANS

by

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INTRODUCTION

Satisfactory preservation of country cured hams and Smithfield type hams depends on dry salting with sodium chloride although other curing and condimental agents such as nitrate, nitrite, sugar, pepper, and other spices contribute to the storage life. Such hams may or may not be smoked before aging for 6 mo. to 2 yrs. In most instances, aging is done in rooms or attics in which temperature and humidity fluctuate. During summer, temperatures of 30 to 35° C usually prevail. Heavy mold growth occurs -- particularly on the flesh side of the ham; some processors regard presence of mold as a sign of proper aging.

Dry European type salamis are made from ground pork and beef and combined in variable proportions with salt, spices, nitrate and nitrite. While the composition of these sausages varies widely, they are of two primary types: (1) Hungarian, in which the salami is cold smoked and (2) Italian, in which no smoke is used. Italian-type salami are hung in "greening" rooms for 4 to 5 days to allow excess moisture to drip from the sausages and for a characteristic mold growth to develop. After smoking, or after growth has started, the salami are moved to ripening rooms where they are aged 1 to 2 mos. under controlled temperature (10 to 15°C) and humidity (75%) conditions. During aging, profuse mold growth usually develops. Mold-ripened sausages are important items of trade on the U. S. West Coast and big city areas. In Europe, mold-ripened salami represent a high percentage of total production.

Isolation of aflatoxin-producing strains of Aspergillus flavus and A. parasiticus from moldy pecans (Carya illinoensis) prompted concern with regard to potential toxin contaminations in processed pecans. While several studies are available concerning measures that effectively control mold growth and toxin contamination during production, storage, shelling and processing, no studies provide data on the frequency of infestation of pecans with A. flavus or A. parasiticus, or of nuts with detectable contents of aflatoxins. Knowledge of these rates of occurrence is a prerequisite to successfully establishing sampling plans for nuts entering a shelling and processing plant and assessing the effectiveness of sorting steps during shelling and processing of pecans.

Investigations of the occurrence of A. flavus and of aflatoxins in peanut and cottonseed lots give evidence that natural toxin contamination occurs only in small proportion of the seeds, but that high toxin concentrations are found in those seeds that are contaminated.

In-shell pecans often are air-classified prior to cold storage, shelling and processing. Pecans with low specific weights, i.e., nuts with shriveled, shrunken or unfavorably developed kernels, are ejected from the processing line and collected as blow-outs or culls. With peanuts, those most likely to be aflatoxin contaminated contain discolored, shriveled or damaged kernels. This fact led us to hypothesize that blow-out pecans might be the main source of fungal infestation and toxin contamination within a lot. This study was undertaken to obtain some idea of mold occurrence and of the possible toxin distribution in lots of pecans that enter a shelling plant.

EXPERIMENTAL

Meat Sampling

Four hundred country-cured hams from 30 different U.S. processors and 40 European type salami from 25 separate sources were investigated. More than 550 swab samples from hams and 180 samplings from salamis were made: 980 mold isolated were selected and 843 identified as to genus and 364 as to species (See slide #).

Molds were recovered from ham and sausage surfaces or interiors by using a variety of media as shown in Slide # . Schema of Raper and Fennell (1965), Raper, Thom and Fennell (1949), and Gilman (1957) were used for identifying genera and species.

Nut Samples

Sixteen lots of improved varieties of pecans from the Georgia, Alabama and Louisiana 1972 crop were classified into the following groups (by weight): sound pecans, #1 blow-outs and #2 blow-outs. Seventy-five nuts from each of the three groups were frozen, crushed and stored at -18°C until aflatoxin analysis. Another 50 in-shell sound nuts and 50 in-shell #1 blow-outs from each lot were randomly sampled and inspected for mold growth and then stored at -18° C until plating for mold count.

All 100 halves of the sound nut and #1 blow-out sample of each lot were screened for visible mycelia under a stereoscopic microscope. Extreme black discoloration and shriveling of kernels were also recorded.

One half-kernel each of the 50 nuts of the sound samples and #1 blow-outs from each lot was surface sterilized, transferred to Petri dishes containing malt extract agar and incubated at 27°C. Mold colonies having morphological characteristics of A. flavus were identified in accordance with Raper and Fennell's classification.

The isolates were screened for mycotoxin production in modified CD solution, in YES medium, and in citrate glucose phosphate glucose medium (CGP)

Those molds showing fluorescence in culture by the method of De Vogel et al. were further screened for aflatoxin production by growing on sterile rice and/or YES at room temperature for 7 days, and then extracting according to the method of Eppley.

Sterigmatocystin was extracted and separated by the method of Stack and Rodricks and a derivative formed by treatment with a 20% suspension of aluminum chloride in ethanol. Quantitative determinations of this derivative were then made by comparing the fluorescence with that of sterigmatocystin standards.

To verify that the fluorescent compounds produced by these organisms were definitely toxins, the compounds were isolated, purified, and separated by using preparative thin-layer chromatography. The concentration of the various spots were determined with a fluorodensitometer by comparing with mycotoxin standards. From producing strains, the chloroform extract was rechromatographed on a preparative scale and the suspect spot removed. The mycotoxin was eluted from the silica gel with chloroform, filtered, and the UV absorption spectra obtained.

The four strains of S. ochraceus were screened for ochratoxin production by the method of Eppley (1968) and their TLC's compared to known ochratoxin standards.

The method of Verrett et al. (1964) was used for bioassay. Chloroform extracts of broth were evaporated to dryness and dissolved in sterile propylene glycol and the solution inoculated into the air sac of eggs. Development of the embryo was observed after 4, 6 and 8 days; all eggs failing to develop were discarded. Also, toxicity of A. wentii isolates was determined by feeding mycelial powder mixed with ground Purina mouse chow to mice in different ratios.

Citrinin was separated from CCl_3 extracts by thin-layer chromatography on Adsorbosil-1 using toluene-ethyl acetate-formic acid 6:3:1 as a developing solvent and intensity of fluorescence compared with that of standards.

A. wentii mycelial powder was ground in a No. 3 Wiley mill and extracted using chloroform in a Soxhlet extractor. Culture filtrates were also used. Fertile eggs were injected with extract or filtrate and incubated. Mouse feeding tests were made with extracts and culture filtrates and with moldy rice and corn and mycelin mixed with ground Purina mouse chow.

Results and Discussion

Molds were recovered from the surface of all sausages and hams, but in the interior of only six hams. Wrapped hams held at ambient temperature and humidity during curing and ripening exhibited abundant mold growth, especially on the meat portions -- less on the skin and

fat portions. There was little mold growth on unwrapped hams held at a low relative humidity during ripening. Similarly, mold-ripened Hungarian-type salami and Italian-type salami produced in the U.S., showed heavy mold growth on the casings. Mold growth on genuine Italian salami was scanty.

Only small populations of Rhizopus, Mucor, Mortierella and Syncephalastrum were recovered from 2% of sausages and hams (Table 2). The low water activity (a_w) of cured, aged meats probably is responsible for their poor growth. Paecilomyces, Oospora, Cladosporium, Alternaria, Epicoccum and Fusarium were recovered exclusively from hams. Cladosporium produced deep-seated "black spots" in about 1/4 of the hams. Penicillium, Asperigillus, and Scopulariopsis were the primary flora found on aged hams and sausages. Species of Scopulariopsis were isolated from 1/2 of the sausages examined--in large amounts from genuine Hungarian salami and Hungarian-type sausages but only two isolations were made from hams; their presence was indicated by white spots on the skin surfaces of hams.

Penicillia were recovered from ca 90% of sausages and hams. On salami they prevailed during the entire ripening period or shared predominance with Scopulariopsis species. The P. janthinellum series were recovered from 3/4 of the fermented sausages where they were present in large amounts on the casings (Table 3). According to Ciegler et al. penicillia produced little mycotixin in sausage but, when grown in YES broth, these fungi elaborated penicillic acid, ochratoxin A, tremortin A, citrinin and patulin. They theorized that inactivation of mycotoxins by SH-group occurred more readily in meat than in artificial media.

On hams, penicillia constituted the primary flora early in the ripening process but later were replaced by aspergilli. P. expansum was isolated from 60% of the hams and 40% of the sausages; on many of these products it was predominant. Cured and aged meats provided a suitable substrate for this xerotolerant species. Hams from one producer were the source of P. commune and P. viridicatum isolated.

Seven strains of P. viridicatum elaborated citrinin when incubated at 25 or 30° C but none below 15°C. At 15°C, 84 to 296 µg of citrinin was detected after 21 days incubation.

Aspergilli were recovered from 90% of the hams but from only 1/3 of the sausages. The A. glaucus group, i.e., A repens, A. ruber, A. amstelodami, etc. were the most prominent. These molds were detected on sausages only if little or no growth of penicillia was present but on hams they were always recovered if the a_w decreased to a suitable level during ripening. Also, the increase in temperature, which occurs after 3 or 4 mos. during ham ripening favors growth of aspergilli. Growth of the A. glaucus group on fully mature country cured hams is substantially affected by the prevailing relative humidity. A. ruber was detected only in small amounts on hams not wrapped in paper and held at 65% R.H., but on wrapped hams held under similar storage conditions, it was abundant. In 1964, Rabie et al. demonstrated that corn inoculated with A. amstelodami was toxic to rabbits and poultry. Not only did the moldy corn appreciably reduce growth rates of ducklings and chickens, it proved to be lethal for rabbits when they are the material. Preliminary results in our laboratory indicate that when mice are fed moldy corn contaminated by A. amstelodami, these animals display nervous symptoms, develop paralysis of the legs, and eventually undergo death.

Four species included within the A. restrictus group were isolated from aged cured hams but not from salami. These molds are strongly xerophilic. The third most common group, A. versicolor, also was recovered exclusively from hams -- frequently from sparse growth.

Eighty-nine cultures of Aspergillus isolated from aged cured meats were tested for toxicity to chick embryos. Two of 22 isolates of A. ruber, 5 of 28 of A. repens, 2 of 12 of A. sydowi, 1 of 12 A. restrictus, 2 of 7 or A. amstelodami, 1 of 2 A. chevalieri and an A. fumigatus isolate exhibited toxicity. Among these fungi the chloroform extract from the growth of A. sydowi isolates showed the greatest toxicity. M12 was the more toxic of the two -- yeilding 0% hatchability of chick embryos with a thousand-fold dilution (.00002 ml) while a 1 to 20 dilution of the XVII/17 culture had 15% hatchability.

When subcultured onto suitable laboratory media, ten of 16 isolates of A. versicolor produced sterigmatocystin (Table 6). Further, when three sterigmatocystin producing isolates were inoculated onto country-cured hams at temperatures which may prevail during aging (i.e., 20 to 28°C) detectable amounts of sterigmatocystin were recovered from the ham slices within fourteen days. Alperden et al. also reported that several A. versicolor isolated produced large amounts of sterigmatocystin in artificial media and small amounts in fermented sausages.

Aspergilli of series other than A. glaucus, A. versicolor and A. restrictus were detected only sporadically; yet, there is special interest in A. flavus (including A. parasiticus), A. ochraceus, and A. wentii due to their association with mycotoxicoses and because they were found growing on a meat substrate, suggest a potential health hazard if given the proper circumstances. Six of eight strains of A. flavus-parasiticus isolated from aged hams and mold-ripened sausages produced chloroform-extractable fluorescent compounds which coincided with aflatoxin standards on TLC plates. Four of these organisms were shown to be toxigenic.

Two strains of A. ochraceus from country cured hams produced ochratoxins A and B on rice, defatted peanut meal and country cured ham. After incubating these organisms on sliced ham at 5, 15, 25, 30 and 37°C for 21 days, very little toxin was recovered from ham stored at 15°C and none at 5 or 37°C. However, there was significant ochratoxin production at 25°C. One-third of the toxin was found in the mycelial mat on the ham surface while the remainder had penetrated into the meat to a distance of 0.5 cm.

Mycelial extracts of an A. wentii strain isolated from country cured hams were highly toxic when inoculated into chick embryos or fed to mice. The minimal amount of the toxin needed for zero egg hatchability was 50 µg/egg. Purification by T.L.C. followed by crystallization yielded orange-red crystals with a m.p. of 285-286°C; chloroform solutions of the crystals had absorption maxima at 270, 295 and 452 nm.

The fact that A. flavus, A. parasiticus, A. ochraceus, A. wentii and P. viridicatum grow and, under suitable conditions produce mycotoxins on country cured hams or Italizn-type salami during storage indicates what more attention must be given to sanitation and proper temperature control. While these fungi occur rarely on fermented sausages, they grow readily on substrates having low water activity when aged cured meats are stored at fluctuating summer temperatures in the Southern U. S. From these findings it is apparent that all undesirable fungal growth on meat products should be avoided. The use of organic acids, or their salts, i.e., acetates and sorbates, has been suggested to reduce mold spoilage. More work of this type needs to be done.

As mentioned earlier, the three air classification categories of pecans differ considerably in weight (density). Sound nut, #1 and #2 blow-outs also differ significantly in percentage of meats.

Mycelia were detected on halves of 9% of sound pecans while for #1 blow-outs visible mycelia appeared in halves of 23%. Also, shriveling and discoloration of kernels occurred more frequently in #1 blow-outs than in sound pecans. Only three of the 48 samples of 75 ground pecans contained detectable amounts (more than 8 ppb) of aflatoxins. Two of 16 lots of halves from 50 sound nuts, and 1 half from 50 #1 blow-outs contained spores or mycelia of A. flavus or A. parasiticus. None of the #2 blow-out samples contained detectable amounts of aflatoxins.

A. flavus and A. parasiticus isolates were recovered from 28 of the sound pecan halves and from 15 of the blow-outs (1.9%). When these two species were analyzed for aflatoxins, only one half-kernel contained more than 20 ppb aflatoxin B_1 or G_1. The concentration in one half-kernel of a sound pecan from lot 1 was 343 ppb of B_1 and 473 ppb of G_1. One of the 16 lots contained aflatoxins in the sound pecan fraction, i.e., those pecans which are shelled and processed for edible purposes. The aflatoxin concentration was lower than that which calls for legal action to-date.

Summary

Toxigenic strains of Aspergillus flavus, A. parasiticus, A. ochraceus, A. wentii, A. veriscolor and Penicillium viridicatum were detected in country cured hams and fermented sausages stored at ambient summer temperatures. It was possible to demonstrate that under suitable conditions, citrinin, sterigmatocystins, aflatoxins, ochratoxins, and A. wentii toxin could be produced. Mycotoxin production occurs when the aging temperature increases to 20 to 30°C but before the available water of the hams becomes too low to permit growth of the toxic molds. Low temperatures (10°C) and low humidities (65 to 70%) during aging as well as high salt concentrations on the hams prevent growth and mycotoxin production. Although molds are common flora on aged and cured meats, the identification of toxigenic strains and the ability to these molds to produce toxins from this source has not previously been reported.

Two lots of sound nuts and one lot of #1 blow-outs contained traces of aflatoxins B_1 and G_1. Fungal mycelium was visible on the surface of 9% of the sound pecan kernels and 23% of the #1 blow-outs. From 2 to 4% (1 or 2 of 50 nuts) of the half-kernels of sound grade and #1 blow-out pecans yielded colonies of Aspergillus flavus and A parasiticus.

MYCOTOXINS: CURRENT PROGRAMS

by

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As you know FDA has 18 field laboratories engaged in the analyses of samples of commodities regulated under the Food, Drug and Cosmetic Act. In addition to a general regulatory capability, the District laboratory at New Orleans has a specialized function, with the responsibility for analyzing most of the samples collected under FDA's mycotoxin programs.

Mold growth with its resultant corresponding toxin can occur during harvesting and/or storage, if the moisture and temperature conditions are conducive to their growth. Removal of mycotoxins is often difficult, expensive and not always effective. Many mycotoxins are heat resistant. Some chemical treatments are effective, such as the use of ammonia to inactivate aflatoxin in peanut meal and cottonseed meal, the latter being the subject of a very fine presentation heard yesterday. Other treatments may destroy the nutritional value of the commodity. While more research is definitely needed in the area of detoxification, the best approach to controlling mycotoxins is prevention. The affected industries have been encouraged to establish and maintain quality control systems adequate to prevent mycotoxin contaminated products from being introduced into the food supply. FDA's responsibilities include maintaining surveillance over those commodities with a demonstrated or suspected potential for mycotoxin contamination and to take regulatory action, as necessary, to remove contaminated products from the market.

Food and feeds contaminated by mold are deemed to be adulterated under Section 402(a) (3) of the FD&C Act, if they consist in whole or in part of any filthy, putrid or decomposed substance. Regulatory action under this section is the simplest basis for action where the determination of "substantial" mold in a food product is made by macroscopic or microscopic observations. The recognition, however, that certain molds growing on foods or feeds can produce toxic and/or

carcinogenic metabolic products (mycotoxins) and that the toxin in feed can be transmitted to edible tissue of the ingesting animal has added a new dimension to the consideration of mold contamination. Foods or feeds found to be contaminated with a mycotoxin, even in the absence of demonstrable mold, are deemed to be adulterated under Section 402(a) (1) of the Act, in that they bear or contain a poisonous of deleterious substance, which may render them injurious to health. This interpretation has been upheld in the courts.

Past surveillance activities have established the potential for aflatoxin contamination in peanuts, Brazil nuts, pistachio nuts, cottonseed, copra, corn and walnuts, pecans and almonds.

Peanuts - Aflatoxin contamination of peanuts is primarily a harvest problem although improper storage conditions can also result in contamination. Most peanuts in this country are sold under a marketing agreement between industry and the USDA. The Peanut Marketing Agreement calls for analysis and certification of each lot of shelled peanuts for aflatoxin content. FDA is provided with a copy of the certificate of analysis and the name of the applicant on each lot found to contain excessive aflatoxin.

Our coverage of processed peanut products is based on the levels of aflatoxin reported in lots of raw peanuts and information about the processor's capability for reducing the levels of aflatoxin during processing.

FDA also has a program for random sampling of peanut products to monitor aflatoxin levels. Our primary concern is the aflatoxin content of the finished product. The first slide shows the results of peanut samples analyzed by MAL from FY 1973-to the present. (A)

Brazil Nuts - There is a USDA-Industry program for the testing of all in-shell Brazil nuts being offered for entry into this country. USDA examines and issues a certificate of analysis for each lot of Brazil nuts entered by importers participating in the program. FDA is notified of all lots exceeding guidelines and detains them without further analysis. Reconditioned nuts are analyzed by FDA, as well as those entries not examined by USDA.

Pistachio Nuts - Almost all of the pistachio nuts offered for entry into this country come through the port of New York. A very high incidence of aflatoxin contamination was found in past years, prompting 100% coverage by FDA, as well as visits to 2 of the 3 major exporting countries (Turkey and Iran) by FDA scientists to provide advice for surveillance and control of aflatoxin contamination at the point of production.

There now exists a working agreement with USDA regarding a voluntary testing program whereby USDA upon request from the importer and at his expense, will test incoming lots of pistachio nuts. The program is similar to the Brazil nut program. FDA examines only reconditioned lots and lots not previously examined by USDA.

Cottonseed - Cottonseed may be used directly in animal feed (mostly dairy cattle feed) or the oil removed and the residual meal used for animal feed. Any aflatoxin carried with the oil will be removed or destroyed in the refining process. However, that portion of aflatoxin carried with the meal will remain as a contaminant. The major danger from use of contaminated feed is the possible transfer of aflatoxin to tissues of the ingesting animals. Aflatoxin in dairy feed will end up as a metabolite, Aflatoxin M, in the cow's milk. Aflatoxin M is as potent a carcinogen as the parent aflatoxin, B_1.

The aflatoxin in cottonseed problem is presently most severe in Arizona but has also been found in some areas of Texas and California. Surveys in FY-71 showed 13 of 31 samples of cottonseed meal and 73 of 88 samples of cottonseed, with excessive amounts of aflatoxin. Most of the cottonseed is used within the state in which it is produced and consequently close contact with appropriate state officials must be maintained. Our Los Angeles Laboratory, which has jurisdiction in Arizona and California do their own analysis on this commodity and I have no available data concerning their activities. Results of samples analyzed by MAL from FY1973 to the present are on the next slide. (B)

Copra - All copra brought into this country is intended for production of coconut oil. As with other refined vegetable oils, the aflatoxins dissolved in the oil are removed during processing. The residual aflatoxin in the meal is considered a hazard in that the major use of copra meal is in dairy feed. There are only two copra processing operations in the United States, one of which exports all the residual meal. The other is in the Los Angeles area and sells most of its meal intrastate. Our Los Angeles District office monitors operation of this plant and analyzes samples appropriately.

Corn - Previous surveillance activities have shown corn to be subject to aflatoxin contamination with the Southeast and Midwest being the major areas involved. Some aflatoxin contamination of corn in the field has been demonstrated but the majority of opinion is that contamination is more likely to occur in storage, particularly if held for a week or more, when the corn has a moisture level of 15-20% and is held at temperatures greater than 75°F.

Corn is also subject to invasion by Fusarium molds both in the field and in storage. High moisture and low temperature are conducive to their growth.

One of the estrogenically active compounds, zearalenone, produced by Fusarium mold has been isolated and studied. Methodology for detection and estimation is available. Another Fusarium, less frequently encountered, is F. tricinctum. This mold produced a potent dermal toxin T-2 that has been implicated in the loss of thousands of lives in Russia from eating moldy grains. Adequate methodology for the detection and estimation of this toxin is not presently available.

A common defect of stored corn, known as "blue-eye" corn, is caused by a group of molds, the most common of which is Penicillium martensii. This mold is a known producer of the mycotoxin penicillic acid. All samples of "blue-eye" corn, collected by USDA graders, were found by FDA to have detectable penicillic acid. The USDA has organized a task force on aflatoxins in corn, composed of research, marketing, extension, commodity stabilization, and education services within the USDA. Liaison between the FDA and the USDA task force is maintained through the Office of the Associate Commissioner for Compliance. A USDA-FDA-Industry joint technical committee has been organized to study sampling, analytical and decontamination problems. The results of corn samples examined by MAL from FY 1973-to the present are shown on the next slides. (C, D)

Almonds, Pecans, and Walnuts - The information derived from past mycotoxin compliance programs indicates that aflatoxin is a potential contamination problem in almonds, pecans and walnuts. Research has been initiated by each of the industry groups involved to determine the source of the contamination so that quality control by the processor can be better directed.

Evidence and theory point to in-shell and nut fragments as most likely and fancy whole nut meats at least likely to be contaminated. Our surveillance this year has been concentrated on in-shell nuts and nut fragments. Results of nut samples analyzed by MAL from FY-1973-to the present are shown on the next slides. (E, F, G)

Dried Fruits (Raisins, Figs) - Available information indicates that dried fruits may be susceptible to aflatoxin contamination. Random sampling was begun in the latter half of last FY. Results of analysis for FY 74/75 to date are shown on the next slide. (H)

Other Mycotoxins -

Barley - In the summer of 1971, 23 of 182 samples of barley grain were found to contain detectable amounts of ochratoxin. Although data is available to conclude that ochratoxin is acutely toxic, this data is not sufficient to make a valid conclusion as to the long term effects of subacute doses of the toxin. Before undertaking any such study, we needed to determine the extent of ochratoxin content in the food supply. Initial efforts were directed towards the incidence of ochratoxin contamination of malted barley and whether the contaminant is carried over into the beer during the brewing process. Laboratory tests demonstrated that ochratoxin was not destroyed in the fermentation process. We subsequently examined one malted barley and one beer sample from each of 125 breweries throughout this country. The results showed no ochratoxin found.

Grain sorghum, Oats, Barley, Wheat and Rye - These "grains" tend to dry rapidly on reaching maturity and after harvest and therefore are not subject to mold invasion to the extent that has been found with corn. Yet the possibility of mold contamination from improper storage practices exists and a number of toxigenic molds are commonly found as a part of the normal mold population on these grains. In addition to aflatoxin, methods have been developed and validated for two more possible toxins, ochratoxin A and sterigmatocystin. Results of analysis of grains for FY 74/75 to date are shown on the next slide. (I)

Apples - A common agent causing rot in apples is the mold Penicillium expansum which produced in considerable quantity the carcinogenic toxin, patulin. It is not unexpected, therefore, to find patulin in some apple products, particularly those made from significant amount of unculled apples.

The results of analysis are shown on the next slide. (J) We are investigating the toxicological significance of these results. Sampling will be discontinued until toxicological information is available to establish procedural guidelines.

Milk Products - Last year we completed a program to examine cottage cheese, non-fat dry milk and evaporated milk for aflatoxin M. This aflatoxin is produced when cows consume food contaminated with aflatoxins and it shows up in the milk. Samples were collected from areas where previous surveys showed a high incidence of aflatoxin contaminated corn, cottonseed and cottonseed meal being used in dairy rations. The results of the survey are on the next slide. (K)

Aflatoxin in Milk Products

Region	#Samp. Exam	#Positive	Range (PPB)
Arizona	16	16	.05 - 0.4
E. Texas	25	3	.05
Carolina/Georgia	7	5	.05 - 0.1

In the coming fiscal year, we will continue to monitor aflatoxin levels in peanut products, corn, cottonseed and cottonseed meal, almonds, pecans, walnuts and figs. If we have developed a guideline for patulin we will monitor patulin levels in apple juice. We may continue to examine "small grains" - grain sorghum, oats, barley and rye for aflatoxin, sterigmatocystin and ochratoxin. In addition, methodology for determining citrinin in these grains is being developed. Should it prove satisfactory we will also monitor that mycotoxin routinely. As new analytical methods are developed, FDA's surveillance will be expanded to include other mycotoxins and other commodities susceptible to mold growth.

The presentations and discussions were summarized by Dr. Flatt. He also thanked all of the participants for a very good conference.

The Workshop adjourned at noon, March 14, 1975.

1975 SAES COLLABORATORS CONFERENCE
Southern Regional Research Center

March 13-14, 1975

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